

Modulation of Streptomycin Killing Rate against Mature *Escherichia Coli* Biofilms in the Presence of Medicinal Plant Extracts*

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Abstract

Background. Medicinal plant extracts exhibiting pro- and antioxidant properties may affect antibiotic-induced killing of biofilm-producing bacteria in both synergistic and antagonistic modes. Better understanding of these alternations is required to adjust antibiotic therapy and herbal medicine in order to exclude unwanted losses of antibiotic efficiency.

Aim: to study modulation modes of streptomycin killing rate against mature biofilms of *Escherichia coli* in the presence of different doses of commonly used medicinal plant extracts.

Materials and methods. Pharmacodynamic parameter killing rate and mass biofilm formation were determined in the presence of streptomycin and medicinal plant extracts.

Results. Synergism was found between 100 mg/ml streptomycin and low doses (0.83 mg of dry herb/ml) of green, black tea, *Arctostaphylos uva-ursi*, *Betula pendula* and *Laminaria japonica* against killing mature biofilms. Alternatively, high doses (6.64 mg of dry herb/ml) of green, black tea and *Vaccinium vitis-ideae* demonstrated antagonism, decreasing killing rate and enhancing biofilm formation. Presumably, high doses of the extracts were sufficient to enhance biofilm formation blocking penetration of streptomycin through enlarged biofilm matrix and diminishing the killing rate.

Conclusions. Widely consumed as soft beverages or for prophylactic purposes green, black tea and *V. vitis-ideae* could promote strong antagonistic effects with streptomycin. These extracts can stimulate biofilm production, making benefit for commensal microbiota, but have clinical relevance due to a significant reduction in the lethal efficiency of streptomycin in biofilms of pathogenic strains. This highlights the need of careful antibiotic prescription scheme adjustment when choosing appropriate combinations of plant extracts and antibiotics to achieve a synergistic effect.

Key words: streptomycin, killing rate, biofilms, medicinal plant extracts

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Изменение скорости бактерицидного эффекта стрептомицина при действии на зрелые биоплёнки *Escherichia coli* в присутствии экстрактов лекарственных растений*

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Резюме

Обоснование. Экстракты лекарственных растений, обладающие про- и антиоксидантным действием, могут оказывать двойственное модулирующее влияние на бактерицидные эффекты антибиотиков.

Цель исследования: изучить модулирующие эффекты водных экстрактов лекарственных растений на скорость бактерицидного эффекта стрептомицина при действии на зрелые биоплёнки *Escherichia coli*.

Методы. Измеряли скорость бактерицидного эффекта и валовое биоплёнкообразование в присутствии различных доз экстрактов и стрептомицина.

Результаты. Выявлен синергизм между 100 мг/мл стрептомицина и низкими дозами (0,83 мг сухого вещества/мл) зелёного, чёрного чая, *Arctostaphylos uva-ursi*, *Betula pendula* и *Laminaria japonica*. В то же время, высокие дозы (6,64 мг сухого вещества/мл) зелёного, чёрного чая и *Vaccinium vitis-ideae* оказывали антагонистические эффекты, подавляя скорость бактерицидного эффекта и стимулируя биоплёнкообразование. В последнем случае, вероятно, подавлялось проникновение стрептомицина в матрикс биоплёнки, что и способствовало снижению скорости бактерицидного эффекта.

Заключение. Широко применяемые человеком в пищу зелёный, чёрный чай и *V. vitis-ideae* снижали бактерицидный эффект стрептомицина. Эти экстракты также стимулировали биоплёнкообразование, что может положительно сказываться на жизнедеятельности нормальной микрофлоры человека, однако может привести к стимулированию толерантности болезнетворных микробов. Выявленные ситуации подавления скорости бактерицидного эффекта в присутствии испытуемых экстрактов указывают на необходимость корректировки схем лечения, включающих совместный прием антибиотиков и фитопрепаратов. Полученные данные представляют практический интерес и нуждаются в дальнейшем изучении.

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Streptomycin is a broad-spectrum aminoglycoside antibiotic, which was found effective in pneumonias, abscesses, peritonitis and other infections, caused by the gram-negative bacteria frequently present in the urinary tract [1]. Broad categories of people, including infants, pregnant women, elderly patients with catheters, diabetes and immunocompromised patients, as well as are at a high risk of development of urinary tract infections [2]. The most common causes of urinary tract infections (UTIs) are associated with biofilm-producing bacteria such as *Escherichia coli*.

While green and black tea are common diet constituents, medicinal plant extracts of *Arctostaphylos uva-ursi* and *Vaccinium* sp. are commonly used in prophylaxis of UTIs, *Betula pendula* leaf extracts are used as diuretic, *Laminaria japonica* is known as a strong bactericidal, anti-inflammatory and immunomodulating agent [3]. Beneficial properties of these plant extracts are often explained by their pro- and antioxidant effect [4, 5]. The idea of involvement of reactive oxygen species into non-specific killing mechanisms by a number of antibiotics has also been discussed [6, 7]. Therefore, modulation of antibiotic action in the presence of redox-active compounds requires comprehensive research. Taking into account a global rise in antibiotic resistant bacteria, such investigations might be helpful in creating powerful antimicrobials and herbal adjuvants increasing efficiency of antibiotic therapy. Strong antimicrobials of plant origin are being frequently characterized [8, 9]. At the same time, a decrease in antibacterial action of aminoglycosides in *Escherichia coli* in the presence of antioxidants has been reported [10], highlighting the complexity of interaction modes of antibiotics and plant substances within a bacterial cell. In this work, we examined how streptomycin killing rate against mature biofilms of *E. coli* might be modulated in the presence of commonly used medicinal plant extracts. As an advantage of the study, we investigated effects of both low and high doses of the plant extracts.

MATERIALS AND METHODS OF RESEARCH

Reagents including antibiotic streptomycin, thiamine, casamino acids, agar, Luria-Bertani broth were from Sigma-Aldrich Chemical Co (St Louis, MO, USA). Other reagents were of analytical grade (Reachim, Russia).

Water extracts of medicinal herbs *Arctostaphylos uva-ursi*, *Vaccinium vitis-idaea*, *Betula pendula*, *Laminaria japonica* were prepared from commercial pharmaceutical preparations (OAO Krasnogorskleksredstva and ZAO Ivan-Chai, Russia) and commercial samples of green and black tea (Greenfield "Golden Ceylon", Greenfield tea Ltd., London, W1U 2HQ, UK). Dry herbs (1 g) were boiled in 30 ml of distilled water in a water-bath during 30 min, cooled and sequentially filtrated through paper and membrane (0.45 µm pore size) filters. This initial extract was concentrated by 8 times using a rotary evaporator IKA RV 10 basic (Germany). The final doses applied in the cultural medium were 0.83 mg of dry herb/ml (initial extract) and 6.6 mg of dry herb/ml (concentrated extract). These doses were chosen as those as the minimal creating a visible effect and the maximal which did not result in precipitation during the incubation period. Fresh extracts were used in all experiments.

The strain of *E. coli* BW25113 was obtained from Keio collection [11]. Bacteria were grown overnight at 37 °C without shaking in minimal M9 medium supplemented with glucose (4 g/l), 0.2 % casamino acids and 10 µg/ml thiamine [12]. After centrifugation at 6000 g for 4 min, the cells were resuspended in fresh M9 to initial optical density at 600 nm (OD_{600}) of 0.1. This culture was transferred to 96-well polystyrene microtitre plates (200 µl per well) and incubated statically at 37 °C for 22 h to obtain biofilms.

Mature biofilms were washed twice with 0.9% NaCl. Then 200 µl of fresh M9 (4 g/l glucose) medium supplemented with 0.2% casamino acids, 10 µg/ml thiamine and 5 µl of extract were added in each well. The plates were incubated at 37 °C with shaking (340 rpm) in Shaker Thermostat Sky Line (ELMI, USA) for 1 hour, after that antibiotic streptomycin 10 µg ml⁻¹ and 100 µg ml⁻¹ (corresponding to 1 minimum inhibitory concentration (MIC) and 10 MIC, respectively) was added and incubation continued further for 2 hours.

In order to determine colony-forming ability (CFU/ml) in biofilms, medium was removed, the biofilms were washed with sterile saline and sonicated by two pulses (37 kHz, 30 W) for 1 min each with pause time of 1 min in a water bath sonicator (Ultrasonic cleaning unit Elmasonic S10 H, Elma, Germany). Then, OD_{600} was measured using xMark™ spectrophotometer (Bio-Rad, USA) and 10-ml drops of serial dilutions were plated on LB-agar. Colonies were counted in 24 h after incubation at 37 °C.

The rate of antibiotic-induced bacterial killing (ψ) was calculated based on the decline of the density of viable bacteria over a defined period, using the equation $\psi = [\ln(N_t/N_0)]/t$, where N_t is the cell density (CFU/ml) at time t ; N_0 is the initial cell density before antibiotic addition, and t is the time in hours (2 hours in the current study) [13].

Mass biofilm formation was monitored using the modified microplate biofilm assay from the methods previously described [14, 15]. Wells of 96-well polystyrene microtiter plates containing mature biofilms resuspended in the fresh medium with/without the extracts were prepared as described above. Control wells contained bacteria-free medium and the extracts only. At time zero and every hour of cultivation OD_{600} of each well was measured, broth was removed and wells were rinsed twice with 200 µl of sterile saline. The wells were air-dried and 150 µl per well of 0.1% crystal violet solution was added for 30 min. Then, the colourant was discarded and the wells were rinsed five times with distilled water. The plates were air-dried for 1 h. To quantify biofilms, 200 µl of 96% ethanol was pipetted into each well. After 5 min, 125 µl of the solution was transferred to a separate plate where the OD_{540} were measured using xMark™ spectrophotometer.

The total biofilm formation (BF) or, the mass of biofilms, was calculated using the formulae: $BF = AB - CW$, where AB is the OD_{540} of stained biofilms and CW is the OD_{540} of stained control wells.

Each result is indicated as the mean value of at least five independent experiments \pm the standard error of the mean (SEM). Significant difference was analyzed by Student's

t-test. A P-value of 0.05 was used as the cut-off for statistical significance. Results were analyzed by means of Statistica 6 (ver. 6, 2001; StatSoft Inc.).

RESULTS AND DISCUSSION

Under our conditions, streptomycin killing rate against mature biofilms of *E. coli* BW25113 grew up in a dose-dependent manner and was equal to -0.17 and -2.39 h^{-1} for 10 and 100 mg/ml streptomycin, respectively. In the presence of the tested medicinal plant extracts, we observed modulation of the killing rates. More remarkable effects were found with 100 mg/ml streptomycin (Fig. 1). Interestingly, different doses of the extracts could cause opposite effects: 0.83 mg/ml extracts of green tea, black tea, *A. uva-ursi*, *B. pendula* and *L. japonica* increased killing rate of the antibiotic by about 25 %. Alternatively, 6.64 mg/ml extracts of green and black tea, *B. pendula* and *L. japonica* decreased the killing rate of streptomycin by about 20 %. High doses of *V. vitis-ideae* reduced the killing rate by 3.5 times. In case of 10 mg/ml streptomycin, the extract of *V. vitis-ideae* demonstrated a dose-dependent protection against killing by antibiotic (data not shown). Generally, synergistic effect between 100 mg/ml streptomycin and low doses of the extracts turned into antagonistic mode when doses of the extracts were increased.

The observed effects might be explained by pro-oxidant activity of green and black tea, *A. uva-ursi* and *V. vitis-ideae*, which also contributed to protection of planktonic cultures against ciprofloxacin and ampicillin but increased killing by kanamycin [16]. Nature of antibiotics is essential when their action is combined with the plant extracts. Specific killing mechanism by streptomycin involves inhibition of protein synthesis. Thus, it is more likely to affect rapidly growing bacterial cultures. As for the biofilms, antibiotic penetration through biofilm matrix might be the limiting factor. Thus, to understand the underlying mechanisms of modulation of streptomycin killing rate in the presence of the extracts we investigated changes in the mass biofilm formation during the incubation period. As it was mentioned in "Materials and methods" section, in the model of our experiment mature biofilms were firstly pretreated with the extracts for 1 h before antibiotic was added. After the first hour, we observed dose-dependent stimulating effects on mass biofilm formation by all the extracts excluding *B. pendula* (Fig. 2 A, B). This coincided with our earlier report about stimulating effects of these extract on biofilm formation in planktonic cultures [5]. Here, maximal stimulation was seen with 6.64 mg/ml *A. uva-ursi* and *V. vitis-ideae* extracts, which enhanced mass biofilm formation up to 5.5 times compared to the untreated biofilms. Strong pro-oxidant activity of the tested extracts could stimulate biofilm production via induction of stress response path-

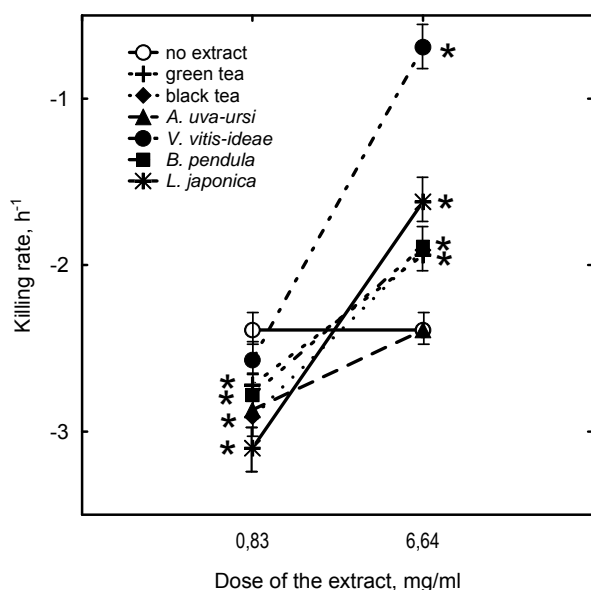


Fig. 1. Killing rate by 100 mg/ml streptomycin in the presence of different doses of the extracts (* indicates statistically significant difference from the sample treated with antibiotic alone).

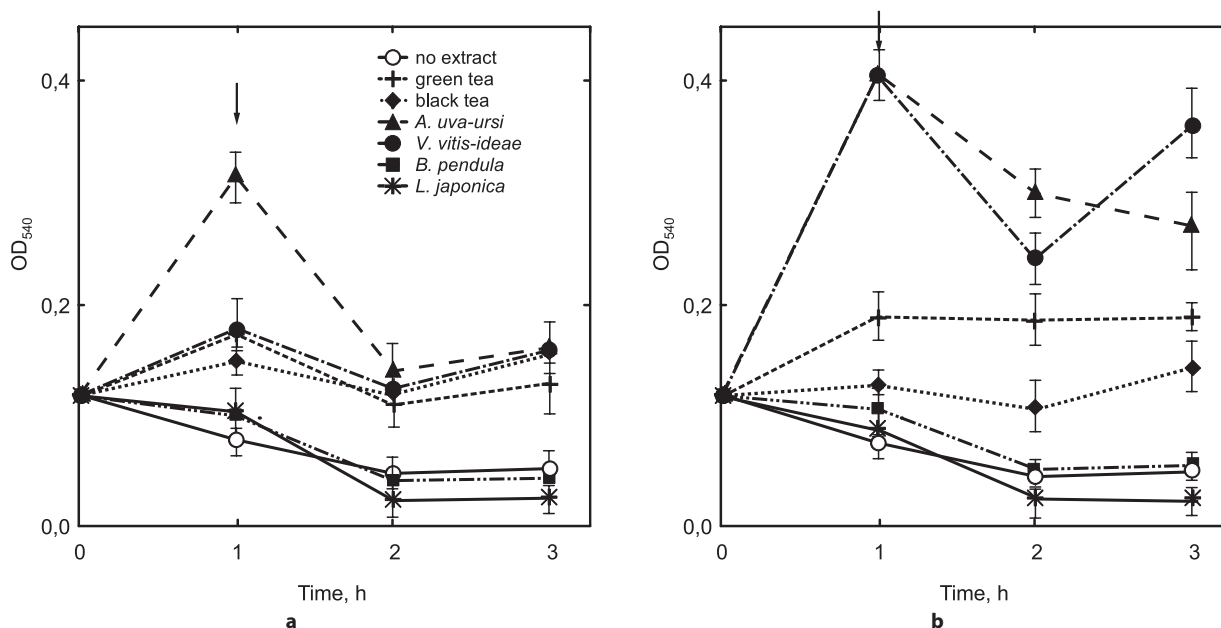


Fig. 2. Mass biofilm formation (OD_{540}) in the presence of low (a) and high (b) doses of the extracts and 100 mg/ml streptomycin (moment of antibiotic addition is indicated by the arrow).

ways [4; 5, 16]. At the same time, mature biofilms that were not treated with the extracts exhibited a slight eradication mode after 1 h of incubation and then the mass biofilm parameter did not change remarkably (data not shown). Treatment with 100 mg/ml streptomycin in the absence of the extracts enhanced eradication by about 50 %. After addition of streptomycin to the biofilms pretreated with the extracts a dose-dependent stimulation of mass biofilm formation was still observed after 2 h of incubation in case of *A. uva-ursi*, *V. vitis-idaea*, green and black tea compared to the sample treated only with antibiotic. Simultaneously, *L. japonica* extract decreased biofilm formation by 2 times (Fig. 2 A, B).

Collectively, after treatment with 100 mg/ml streptomycin high doses of the extracts of green, black tea and *V. vitis-idaea* demonstrated antagonistic mode, decreasing antibiotic killing rate and enhancing biofilm formation. At the same time synergism was found between streptomycin and low doses of the extracts of green tea, black tea, *A. uva-ursi*, *B. pendula* and *L. japonica* against killing mature biofilms. Apparently, high doses of the extracts were sufficient to enhance biofilm formation and consequently to block penetration of streptomycin through enlarged biofilm matrix and thus the killing rate was diminished.

CONCLUSION

Our findings revealed opposite modulation patterns of the streptomycin killing rate in the presence of low and high doses of the medicinal plant extracts. Notably, only low doses provoked synergism enhancing killing of mature biofilms by streptomycin. Higher doses of green, black tea and *V. vitis-idaea*, which are widely consumed as soft beverages or in UTIs prophylaxis aroused strong antagonistic effects decreasing killing by streptomycin. We showed that these extracts could stimulate biofilm production, which may be useful for commensal microbiota, but have clinical relevance due to a significant reduction in the lethal efficiency of streptomycin in biofilms of pathogenic strains. This highlights the need of carefulness and antibiotic prescription scheme adjustment in choosing appropriate combinations of plant extracts and antibiotics to achieve a synergistic effect. Further research is required to elucidate the complicated interaction patterns of antibiotics and medicinal plant extracts.

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Conflict of interest

The authors have no conflict of interest to disclose.

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