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Full Length Research Paper

The effect of propolis on microbial vitality and oxygen consumption

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The aim of this study was to investigate the effect of different concentrations of ethanolic extract of propolis (EEP) on oxygen consumption and vitality of gram-positive, Gram-negative bacteria, and yeast. Our investigations showed that EEP at the concentration of 65 - 1,300 μg ml^{-1} of phenolic compounds (PC) inhibited oxygen consumption rate of *Bacillus subtilis* by 26 - 81%, and of *Candida albicans* - by 6 - 70%, and significantly decreased cell count of the investigated Gram-positive bacteria (*Bacillus cereus, B. subtilis*) and yeast. EEP at the concentration of 260 - 2,340 μg ml^{-1} of PC inhibited oxygen consumption rate of *Escherichia coli* by 6 - 54%, and reduced cell count of Gram-negative bacteria (*Salmonella typhimurium, E. coli*). Positive linear correlations (r^2 - 0.97, 0.98, and 0.89 with *B. subtilis*, *C. albicans* and *E. coli*, respectively) were revealed between the inhibition of oxygen consumption and vitality of the investigated microorganisms. Therefore, it is believed that EEP inhibition of oxygen consumption decreases the vitality of microorganisms, and it could be one of the reasons for the antimicrobial action of propolis.

Key words: Ethanolic extract of propolis, phenolic compounds, microbial oxygen consumption, microbial vitality.

INTRODUCTION

Propolis is a complex resinous material produced by honeybees from plant exudates, beeswax, and bee secretions (Drago et al., 2007). The chemical composition of propolis is highly variable, depending on the botanical origin and the season of collection (Marcucci et al., 2001; Kartal et al., 2002). The main components presented in each sample of propolis are phenolic compounds (flavonoids, phenolic acids and their esters), aliphatic acids and their esters, aromatic acids ant their esters, terpenoids (Kujumgiev et al., 1999). However, various samples of propolis differ in both compositions of the active substances and the amounts of these substances, which results in different biological effect (Bankova et al., 2002; Moura et al., 2009).

Nowadays propolis is widely used due to antimicrobial, antiinflammatory, anesthetic, antioxidant, anticarinogenic, vaso-protective, and hepato-protective activities (Mirzoeva and Calder, 1996; Burdock, 1998; Isla et al., 2001; Christov et al., 2006). Numerous authors investigated antibacterial, antifungal, antiviral, and antiprotozoan effect of propolis (Grange and Davey, 1990; Abd El Hady and Hegazi, 2002; Stepanović et al., 2003; Lu et al., 2005; Silici and Kutluca, 2005; Drago et al., 2007; Kumar et al., 2008). It was reported that the antimicrobial activity of propolis collected in middle latitude areas is attributed to the effect of flavonoids, phenolic acids and their esters; in propolis collected in tropical regions, these compounds were either absent or present in minimal amounts, and thus the antimicrobial activity of such propolis is based on the presence of diterpenes and prenylated compounds (Kujumgiev et al., 1999). However, some investigations confirm the fact that propolis, independently of its plant source and chemical composition, always possesses antimicrobial properties, even though different chemical constituents are responsible for it (Trusheva et al., 2006). Moreover, antimicrobial effect is likely to be caused by multiple

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mechanisms and synergistic effect of flavonoids, terpenoids, phenolic acids and other compounds presented in propolis. Several mechanisms of this activity have been reported: (1) inhibition of cell division; (2) collapse of cell membranes and walls (3), and inhibition of protein synthesis (Fernandes Junior et al., 2005). Nevertheless, the exact mechanism of its action is not well known.

In our previous studies we investigated the effect of aqueous solution of propolis on mitochondria that supply a cell with energy (Majiene et al., 2006). The results of our study showed that the active substances of propolis in a dose-dependent manner have an inhibitory effect on mitochondrial respiration. Since the mechanism of energy production in mitochondria is similar to that in aerobic microorganisms, we hypothesized that propolis might inhibit microbial respiration. There are no data on the action of propolis on microbial oxidative metabolism. Thus, the aim of this study was to investigate the effect of different concentrations of EEP on oxygen consumption and vitality of Gram-positive and Gram-negative bacteria and yeast.

MATERIALS AND METHODS

Quantitative analysis of propolis raw material and EEP

Propolis was collected in Lithuania during July - September of 2007. The hive from which propolis was collected was surrounded with meadows and leafy forest. The collected propolis sample was stored in a dark and dry place. The total amount of propolis raw material, phenolic compounds, total amount of flavones and flavonols, and total amount of flavanones and dihydroflavononones was determined as described earlier (Popova et al., 2004).

Ethanolic extract of propolis (EEP) was prepared from propolis that before the extraction was cooled, grated, and extracted with 70% ethanol (1:10) at room temperature for 24 h. The content of total phenolic compounds in EEP was determined as described earlier (Savickas et al., 2005).

Test microorganisms

Antimicrobial activity of propolis was established for 5 test strains of microorganisms: Gram-positive bacteria spore-forming bacteria (*Bacillus cereus* ATCC 11778, and *Bacillus subtilis* ATCC 6623,), Gram-negative bacteria (*Salmonella typhimurium* ATCC 14028, *Escherichia coli* β-lac(-) ATCC 25922), and yeast (*Candida albicans* ATCC 60193).

Determination of the vitality of microorganisms

Bacterial cultures were grown for 18 h at the temperature of 37°C on slant agar (CM 325, *Oxoid*). The grown cultures were washed off the agar using sterile saline solution, and cell suspensions were adjusted according to McFarland No. 0.5 standard (NCCLS, 1998).

The yeast fungal culture grew for 48 h at the temperature of 25°C on slant yeast extract glucose chloramphenicol agar (YGC Agar, *Merck*). The grown cultures were washed off the agar using sterile saline solution. The cell suspension prepared according to McFarland standard No. 3 was poured into dissolved and cooled to 45°C yeast extract glucose chloramphenicol agar.

Then 0.01 ml of each cell suspension was introduced into 10 ml of Brain Heart Infusion broth (No. 610008, *Liofilchem Diagnostici*). Different amounts of the EEP were added to the broth. A control sample was prepared by introducing 0.01 ml of the suspension of the respective test culture into 10 ml of the broth. The prepared samples were stored at room temperature (20°C). The number of test culture cells in the broth was determined using the Petri dish technique and a growth medium (CM0325, *Oxoid*). The dishes were incubated for 48 h at the temperature of 30°C. The antimicrobial activity was evaluated by comparing the cell count in the tested samples; in the growth medium without EEP (control sample), and in growth media with various amounts of EEP. The cell counts were determined immediately after stirring and after 24 h. If no bactericidal effect was detected, and the growth of a certain number of cells was found, the effectiveness of inhibition was calculated, that is by how many times the number of viable cells detected in the tested samples was lower than that in control sample. Control experiments with a solvent used at the same amounts as EEP had no effect on the vitality of the studied microorganisms.

Measurement of oxygen consumption

0.01 ml of the suspension of the respective test culture was introduced in a solution containing 20 mM of imidazole, 20 mM of taurine, 0.5 mM of dithiothreitol, 1.6 mM of $MgCl_2$, 100 mM of MES, 3 mM of KH_2PO_4, 3.0 mM of CaK_2EGTA, and 7.1 mM of K_2EGTA (free Ca^{2+} concentration was 0.1 μM) (pH 7.1 adjusted with KOH at 37°C). Oxygen uptake rates were recorded at 37°C using the Clark-type electrode system. The solubility of oxygen was estimated to be 422 nmolO/ml. Substrates used in the measurements were 4 mM of glutamate + 4 mM of malate.

Statistical analysis

Data are presented as means ± S.E.M. Nonparametric methods were applied for making inferences about the data. Differences between mean values in dependent groups were tested using Wilcoxon matched pairs test. Differences between mean values in independent groups were tested using nonparametric Kruskal-Wallis test with Dunn's post-hoc evaluation. The statistical analysis was performed at $p < 0.05$ using the software package Statistica 1999, 5.5 StatSoft Inc., USA.

RESULTS AND DISCUSSION

In the present study we used propolis raw material collected from Lithuania; the total amount of phenolic compounds was 21.7 ± 0.3%, the total amount of flavones and flavonols was 2.6 ± 0.3%, and the total amount of flavanones and dihydroflavononones was 5.4 ± 0.6%. Compounds detected in this propolis are characteristic of *Populus tremula,* from which the bees seem to have collected balsams.

In the first experiment it was investigated the effect of EEP at the concentration of 65 - 2,600 μg ml^{-1} of PC on the vitality of microorganisms grown for 24 h in the Brain Heart Infusion broth. The obtained findings showed that 65 μg ml^{-1} of PC in the growth medium significantly decreased the number of cells of *Bacillus cereus* (Figure 1A), *B. subtilis* (Figure 1B), and *Candida albicans*

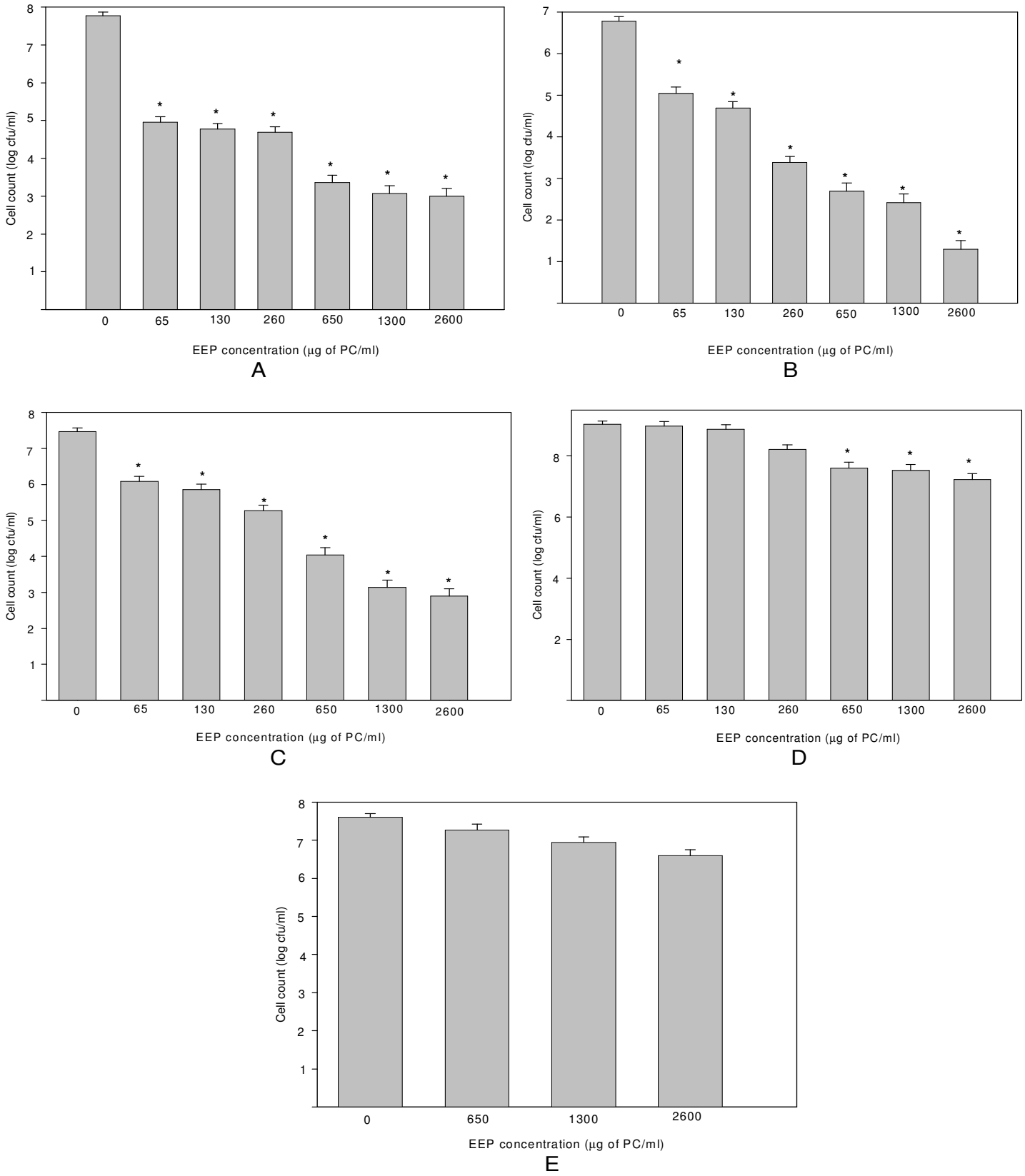

Figure 1. The effect of different concentrations of EEP on the susceptibility of microbial strains. A-*Bacillus cereus*, B-*Bacillus subtilis, C-Candida albicans*, D-*Escherichia coli*, E-*Salmonella typhimurium*. *P < 0.05 *vs*. cell count in growth medium without EEP. Number of experiments-3.

(Figure1C). The growth of gram-negative bacteria was either unaffected at such concentration of the EEP (*Salmonella typhimurium,* Figure 1E) or the effect was insignificant (*Escherichia coli,* Figure 1D). Higher concentration of EEP (130 - 2,600 μg ml^{-1} of PC) had a higher inhibitory effect on the growth of the studied microorganisms. Thus, investigations of microbial growth in media with different amounts of EEP showed that gram-positive spore-forming bacteria (*B. cereus* and *B. subtilis*) and yeast (*C. albicans*) were sensitive to the effect of propolis already at the lowest investigated concentration of EEP (65 μg ml^{-1} of PC). Meanwhile, EEP started reducing the growth of Gram-negative bacteria only at the concentration of 260 - 650 μg ml^{-1} of PC.

It is difficult to compare our findings with those obtained by other researchers since the chemical composition and concentrations of various propolis preparations used by different authors are different and differently expressed, therefore the obtained results demonstrate that propolis is active against bacteria at different orders of magnitude (mg ml^{-1} or μg ml^{-1}) (Abd El Hady and Hegazi, 2002; Savickas et al., 2005). Several studies demonstrated that antibacterial properties of propolis were attributable to its high flavonoid content (Grange and Davey, 1990; Burdock, 1998). Although the concentration of flavonoids was lower in our propolis sample, compared to propolis collected in other European countries, even at concentrations of μg ml^{-1}, it effectively reduced the vitality of the studied microorganisms. Thus, our investigations confirm the opinion of some authors who state that the mechanism of antimicrobial activity of propolis is complex and could be attributed to the synergistic activity of flavonoids and other polyphenolic compounds.

Our further experiments were performed to investigate the effect of different concentrations of EEP on oxidative metabolism of microorganisms. At the beginning we measured the respiratory rate of *Bacillus subtilis*, which is one of the most sensitive bacteria to the effect of EEP. Figure 2A presents a typical polarographic curve of oxygen consumption by *B. subtilis* (addition O - A1). After 3 min we added EEP (65 μg ml^{-1} of PC) into the respiration medium. This concentration of EEP inhibited oxygen consumption of the investigated bacteria by 26.2%.

By further increasing the concentration of EEP (130 - 975 μg ml^{-1} of PC), the oxygen consumption was inhibited by 41 - 81%. In parallel, we investigated the effect of different concentrations of the antibiotic gentamycin (2 - 16 μg ml-1) on the respiratory rate of *B. subtilis*. Figure 2B presents polarographic curve which demonstrate that gentamycin had no effect on the oxygen consumption of the studied bacteria irrespectively of the concentrations used (additions A1 - 8). It is known that the antimicrobial effect of gentamycin is due to the inhibition of glycoprotein synthesis in the walls of bacteria, and therefore the effect is seen not immediately after the addition of the drug. Differently from gentamycin, active substances in the EEP immediately inhibited the oxidative phosphorylation of the investigated bacteria (additions A9, A10).

In further experiments we used the same protocol to determinate the effect of EEP on oxygen consumption of *Candida albicans* and *E. coli* (Table 1). The obtained data showed that EEP at the concentration 65 - 1,300 μg ml^{-1} of PC inhibited oxygen consumption rate of *C. albicans* by 6.4 - 69.6%, and at the concentration of 260 - 2,340 μg ml^{-1} of PC inhibited oxygen consumption rate of *E. coli* by 5.9 - 53.6%.

In order to evaluate relationship between the inhibition of microbial oxygen consumption and the decrease in the vitality of the investigated microorganisms, we revealed positive linear correlations (R^2 -0.97, 0.98, and 0.89 with *B. subtilis*, *C. albicans* and *E. coli*, respectively) indicating that is a close agreement of investigated parameters. These high correlations may suggest that EEP inhibition of oxygen consumption can decrease microbial vitality.

Mirzoeva and coworkers showed that propolis flavonoids (pinocembrin, galangin, and pinobanksin) uncouple the energy transducing cytoplasmic membrane and inhibit bacterial motility (Mirzoeva et al., 1997). We failed to find any more results in literature about the effect of propolis on bacterial energy production. However, it has been demonstrated that phenolic acids inhibit microbial enzymes, depriving microorganisms of the substrates required for microbial growth or via direct action on microbial metabolism through oxidative phosphorylation (Nohynek et al., 2006). Moreover, our earlier investigations revealed that water solution of propolis inhibited the basal and maximal (ADP-stimulated) respiration rate of rat heart mitochondria respiring on pyruvate+malate and succinate (Majiene et al., 2006).

The results of this study for the first time demonstrated that EEP inhibited oxygen consumption of the investigated bacteria and yeast respiring on glutamate+malate. It is known that inhibition of respiration results in inhibition of phosphorylation, which reduces the amount of ATP. Since the microorganisms use energy concentrated into the bond of ATP for their growth and reproduction (Tortora et al., 2004), the inhibition of ATP synthesis could be one of the reasons for the antimicrobial action of propolis.

Conclusion

EEP in a dose-dependent manner inhibited microbial respiration and decreased vitality in our investigated aerobic microorganisms. Positive linear correlations (R^2-0.97, 0.98, and 0.89 with *B. subtilis*, *C. albicans* and *E. coli*, respectively) were revealed between inhibition of oxygen consumption and vitality of the investigated microorganisms. We assume that EEP caused inhibition of microbial oxygen consumption can decrease vitality of microorganisms.

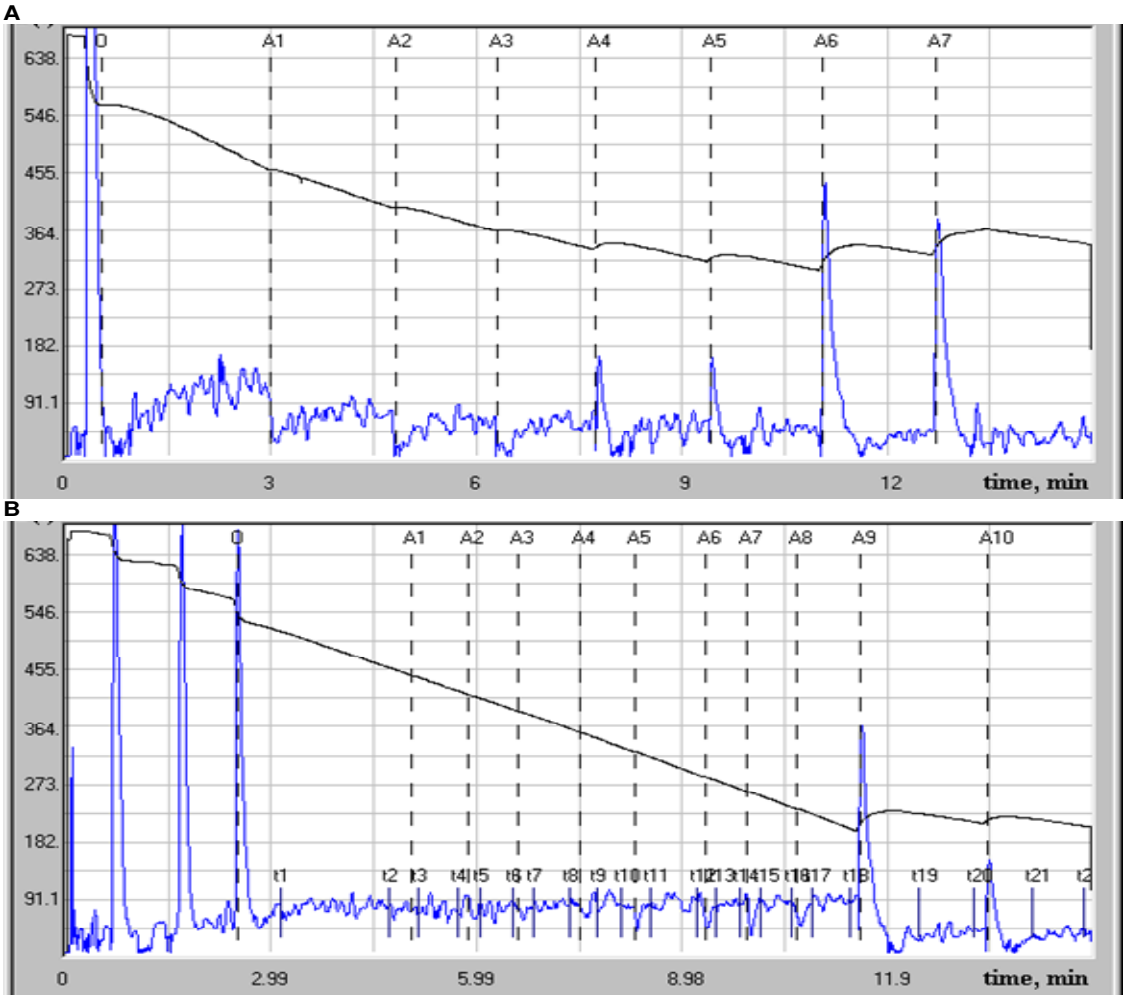

Figure 2. Original traces of *Bacillus subtilis* respirometric measurements. Standard incubation medium was supplemented with 4 mM of glutamate + 4 mM of malate. Additions: 0-0.01 ml of *Bacillus subtilis* suspension. (A) A1, A2, A3-each of 65 µg/ml of PC; A4, A5-each of 130 µg/ml of PC; A6, A7-each of 260 µg/ml of PC. (B) A1, A2, A3, A4, A5, A6, A7, A8-each of gentamycinum 2 µg/ml, A9 , A10-each of 65 µg/ml of PC.

Table 1. The effect of different concentrations of EEP on oxygen consumption of *Candida albicans* and *E. coli*. Measurements were performed in the presence of 4 mM of glutamate + 4 mM of malate. Higher amounts of EEP were not used due to inhibitory effect of 70% ethanol on oxygen consumption in the investigated microorganisms. *- $p < 0.05$ *vs.* oxygen consumption rate without EEP. Number of experiments - 5.

	EEP µg/ml of PC	Inhibition of oxygen consumption, %
Candida albicans	65	-6.4 ± 2.1
	130	-12.5 ± 3.1
	260	-30.4 ± 3.2*
	520	-42.7 ± 0.6*
	780	-59.3 ± 5.0*
	1300	-69.6 ±1.5*
E. coli	260	-5.9 ± 2.0
	780	-14.1 ± 7.0
	1300	-31.8 ± 13.0*
	1820	-48.4 ± 6.2*
	2340	-53.6 ± 3.7*

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