UNIVERSITY OF PARDUBICE

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Department of Biological and Biochemical Sciences

METHODS FOR MONITORING OF ANTIMICROBIAL ACTIVITIES OF NATURAL COMPOUNDS

Annotation of Ph.D. Degree Thesis

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LIST OF ABBREVIATIONS

BHI	Brain-Heart Infusion
BTS	Beltsville Thawing Solution
CCM	Czech Collection of Microorganisms
CFU	Colony Forming Unit
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
FBCI	Fractional Bactericidal Concentration Index
FIC_A	Fractional Inhibitory Concentration of substance A
FIC_B	Fractional Inhibitory Concentration of substance B
FICI	Fractional Inhibitory Concentration Index
MBC	Minimum Bactericidal Concentration
MBC_{50}	Minimum Bactericidal Concentration for 50% of bacterial cells
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MIC_{50}	Minimum Inhibitory Concentration for 50% of bacterial cells
Ps. aeruginosa	*Pseudomonas aeruginosa*
St. aureus	*Staphylococcus aureus*

INTRODUCTION

Several hundred million insemination doses are produced annually as part of the worldwide insemination process. The parameters of boar ejaculates and the insemination doses prepared from them are subject of high requirements, including their health safety. Among important factors reducing the quality of boar ejaculates intended for artificial insemination is microbial contamination. Microorganisms have adverse effects on sperm cells and may also contribute to the development of inflammatory changes in the genital tract of sows. Antibiotics or combinations of antibiotics are used to inhibit microbes in insemination doses. However, these drugs are primarily intended for therapeutic purposes. It is therefore necessary to look for other possibilities for the decontamination of insemination doses, since the use of antibiotics for these purposes contributes to increasing the number of resistant microbial strains.

One of possible solution is to use antimicrobial effects of natural substances derived from natural sources, mainly including plants. For centuries, natural substances are obtained from different plants species due to their antibacterial, antifungal, antiviral, anti-inflammatory and antioxidant properties (Maher *et al*., 2012). Significant decrease in natural substances use had been mentioned since the 1950s as a result of development of antibiotic therapy. Trust in antibiotics and their overuse have resulted in the progressive resistance of microorganisms to these drugs. Besides, the development of new antibiotics is relatively slow.

This serious worldwide problem requires the search of new compounds with antimicrobial activities. One of the alternatives may be natural substances, which are mostly well available from many plants, relatively cheap and no significant resistance have been documented so far.

1 AIMS

The claim mentioned above had been main reason for verification of antimicrobial activities of selected natural substances in our study. First, it was necessary to select suitable methods for detection and quantification of microorganisms in boar semen samples, then to select methods for natural substances antimicrobial activities assessment. Microdilution, dilution and agar dilution method were verified and evaluated for these purposes.

The tested substances were thymol, carvacrol, eugenol, gallic acid, four gallic acid esters, cnicin, usnic acid, and others not mentioned in the annotation. We also examined their mutual combinations and combinations with EDTA in order to find useful interactions among them. We used the FICI (Fractional Inhibitory Concentration Index) and FBCI (Fractional Bactericidal Concentration Index) to determine interactions between double combinations of natural substances. For simplification due to the high number of results, we used MIC_{50} and MBC_{50}. Important part was the verification of toxicity of natural substances for boar sperm cells. Final part was the assessment of the possibility of using natural compounds for the decontamination of boar semen. These aims were solved within the dissertation work.

1. Introduction of methods for qualitative and quantitative detection of microorganisms in model biological material
2. Introduction and selection of methods for the determination of antimicrobial effects of natural substances
3. Determination of the antimicrobial effects of natural substances and their selected double combinations on microorganisms
4. Introduction of a method to detect the toxicity of selected natural substances for boar sperm cells
5. Analysis of the results and selection of suitable substances or their combinations for the boar semen decontamination

2 MATERIAL AND METHODS

2.1 MICROOGANISMS USED FOR ANTIMICROBIAL ACTIVITIES TESTING OF NATURAL SUBSTANCES, ESSENTIALS OILS AND OTHER COMPOUNDS

The antimicrobial effects of natural substances and their combinations were examined on bacterial and yeasts strains obtained from the Czech Collection of Microorganisms (CCM) in Brno, from the collections of the General University Hospital in Prague and strains isolated from boar ejaculates in the microbiological laboratory of the Department of Biological and Biochemical Sciences of the University of Pardubice.

Reference microorganisms from the CCM used for evaluation of antimicrobial activities of natural substances were as follows: *Bacillus subtilis* (CCM 2215), *Enterococcus faecalis* (CCM 4224), *Listeria monocytogenes* (5576), *Staphylococcus aureus* (CCM 4223), *Alcaligenes faecalis* (CCM 1052), *Escherichia coli* (CCM 3954), *Pseudomonas aeruginosa* (CCM 3955), *Bacteroides fragilis* (CCM 4508), *Candida albicans* (CCM 8186).

Microorganisms isolated from boar semen used for evaluation of antimicrobial activities of natural substances were as follows: *Enterococcus durans* (n = 2), *Enterococcus faecalis* (n = 2), *Streptococcus porcinus* (n = 4), *Escherichia coli* (n = 4), *Proteus mirabilis* (n = 7), *Providencia stuartii* (n = 2), *Pseudomonas aeruginosa* (n = 17), *Candida catenulata* (n = 1).

Other microorganisms used for evaluation of antimicrobial activities of natural substances were as follows: *Enterococcus faecalis* (n = 5), *Enterocococcus faecium* (n = 8) and *Enterococcus gallinarum* (n = 1) from *General University Hospital in Prague and Klebsiella pneumoniae* (n = 1), *Clostridium perfringens* (n = 1) and *Rhodotorula rubra* (n = 1) from KBBV collection.

2.2 CHEMICALS

2.2.1 TESTED COMPOUNDS

Natural substances tested in our work were thymol, carvacrol, eugenol, gallic acid, methyl gallate, ethyl gallate, propyl gallate, octyl gallate, knicin, usnic acid, α-pinene, β-pinene, limonene, berberine, rosmarinic acid, *p*-cymene. Examples of selected natural substances structures, see Figure 1 – 4. Essential oils tested were *Carvi aetheroleum, Foeniculi aetheroleum, Lavandulae aetheroleum, Rosmarini aetheroleum, Terebinthinae aetheroleum*. Other compound was EDTA. Solvents used for dissolving of natural substances were ethanol and DMSO.

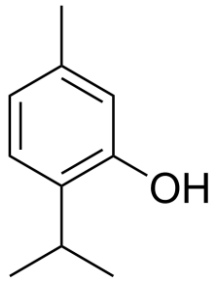

Figure 1: Structure of thymol [1]

IUPAC name: 5-methyl-2-propan-2-ylphenol

Molar mass: 150,22 g.mol^{-1}

Molecular formula: $C_{10}H_{14}O$

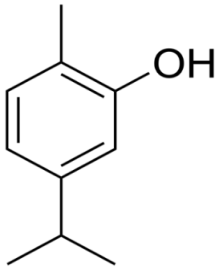

Figure 2: Structure of carvacrol [2]

IUPAC name: 2-methyl-5-propan-2-ylphenol

Molar mass: 150,22 g.mol^{-1}

Molecular formula: $C_{10}H_{14}O$

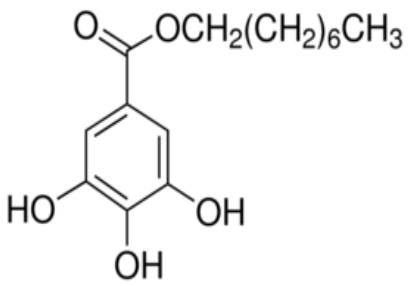

Figure 3: Structure of octyl gallate [3]

IUPAC name: octyl-3,4,5-trihydroxybenzoate

Molar mass: 282,34 g.mol^{-1}

Molecular formula: $C_{15}H_{22}O_5$

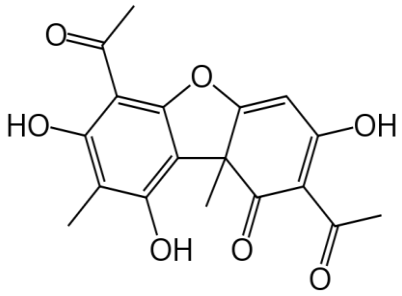

Figure 4: Structure of usnic acid [4]

IUPAC name: 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3-dione

Molar mass: 344,32 g.mol^{-1}

Molecular formula: $C_{18}H_{16}O_7$

2.3 PROCEDURE FOR THE EXAMINATION OF MODEL BIOLOGICAL SAMPLES

2.3.1 METHOD FOR QUALITATIVE EXAMINATION

Samples of native boar semen were obtained from the Research Institute of Animal Production in Kostelec nad Orlicí. In total, 30 samples were examined, which were transported chilled to a temperature of 17°C and processed no later than 4 hours after the collection. Before cultivation, the samples were homogenized in a shaker. Ejaculates were cultured on common and selective-diagnostic media and further in broth for multiplication of microorganisms. All media and solutions were let to reach the room temperature before use. 100 μL of boar semen was pipetted into each medium and spread homogenously on sterile medium with a sterile loop. The growth of microbes was evaluated after 24 and 48 hours of incubation under aerobic condition at 37°C. Isolated strains were identified based on growth, biochemical, morphological and antigenic properties.

2.3.2 METHOD FOR QUANTITATIVE EXAMINATION

For determination the total number of bacteria and yeasts, semen was diluted with sterile physiological saline. An undiluted ejaculate, 10x, 100x, and 1000x diluted ejaculate was prepared. From each dilution 1 mL of semen was transferred to the bottom of the Petri dish and then freshly sterilized Mueller Hinton Agar cooled to 40 – 45°C was added. After solidification, the media was placed into incubator and incubated for 24 and 48 hours under aerobic condition at 37°C. The colonies were counted in all dilutions and the total numbers of microorganisms in 1 mL of semen were evaluated.

2.4 METHODS FOR DETERMINATION OF ANTIMICROBIAL ACTIVITES OF NATURAL SUBSTANCES

2.4.1 MICRODILUTION METHOD

Preparation of stock solution of tested substances

Natural substances were dissolved in 96% ethanol and then Mueller Hinton Broth (MHB) was added. Concentration of ethanol did not exceed 1% after final preparation.

Preparation of microbial suspension

Bacterial suspension was prepared in physiological saline, density was determined using nephelometer. Final density was adjusted on $1.5x10^8$ $CFU.mL^{-1}$ in case of bacteria and on $1.5x10^6$ $CFU.mL^{-1}$ in case of yeasts.

Preparation of dilution range for determination of antimicrobial activities of tested substances

To each well of the first column (series A – H) of the sterile microtiter plate was pipetted 270 μL of stock solution of natural substance and 135 μL of MHB into individual wells of columns 2 – 12 (series A – H). The substances were diluted with use of two-fold dilution, we mostly used concentration range of 4.7 – 4800 $\mu g.mL^{-1}$.

In this way, a two-fold dilution of the test compounds with 10 or 12 different concentration was prepared (the concentration range was slightly different for each substance). Subsequently, 15 μL of the microbial suspension was added to each well. Microtiter plate was incubated under aerobic condition for 24, 48 and 72 hours at 37°C. All substances were tested in triplicate. All determination of antimicrobial activity included the control of growth of microorganisms in MHB without inhibiting substances, without solvent and control of medium sterility.

Evaluation

The minimum inhibitory concentration (MIC) was evaluated as the dilution of the natural compound in the first well without visible growth of microorganisms. To determine the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC), the content of the last well with visible growth and at least 3 wells without visible growth were inoculated on blood agar and incubated for 24 – 48 hours at 37°C. The concentration

of the first compound, where the microbial growth was not observed on blood agar was assessed as MBC or MFC.

2.4.2 BROTH DILUTION METHOD

Preparation of stock solution of tested substances (according to chapter 4.1)

Preparation of microbial suspension (according to chapter 4.1)

Preparation of dilution range for determination of antimicrobial activities of tested substances

Natural substances were diluted using two-fold dilution with final volume 1 mL after addition of 100 μL microbial suspension. One substance was tested twice-time at the same time. All analysis of antimicrobial activity included the control of growth of microorganisms in MHB without inhibiting substances, without solvent and control of medium sterility.

Evaluation (according to chapter 4.1)

2.4.3 AGAR DILUTION METHOD

Preparation of stock solution of tested substances

Natural substances were dissolved in 96% ethanol and then sterilized Mueller Hinton Agar cooled on 40 – 45°C was added. Concentration of ethanol did not exceed 1% after final preparation.

Preparation of microbial suspension (According to chapter 4.1)

Suspension was added on marked area of medium with use of sterile calibrated 10 μl loop.

Preparation of dilution range for determination of antimicrobial activities of tested substances

For dilution range of natural substance consisting of 10 concentrations, the two-fold dilution was used. The stock solution of each natural substance concentration was added to sterilized Mueller Hinton Agar cooled on 40 – 45°C and mixed properly. The mixture was added into Petri dish and let solidified. Subsequently, medium was divided to 16 same parts and into each part, the microbial suspension was added (one medium = one concentration). Media were incubated 24, 48 and 72 hours in 37°C under aerobic condition. Experiments were performed in triplicates.

Evaluation

MIC was evaluated as the first medium with some concentration of natural compound, where the growth was not observed.

2.5 METHOD FOR DETERMINATION OF ANTIMICROBIAL ACTIVITES OF NATURAL SUBSTANCES IN COMBINATION

2.5.1 MICRODILUTION METHOD

Preparation of stock solution of natural substances

The first natural substance was dissolved in 96% ethanol and then MHB was added. The second natural substance was prepared in the same way but 15 times concentrated than the first substance.

Preparation of microbial suspension (According to chapter 4.1)

Preparation of dilution range for determination of antimicrobial activities of natural substances double combinations

Into wells of microtiter plates with the exception of column 1 was pipetted 125 μL of MHB. The wells of column 1 (A – H) were filled with 250 μL stock solution of the first compound. The concentration of the first substances was 1.2 times higher than the required due to dilution after the addition of 10 μL of the second natural substance and 15 μL of the microbial suspension. Subsequently, 125 μL from the stock solution of the first compound was pipetted by two-fold dilution into the wells of column 2 (A – H). After thorough mixing, 125 μL of the mixture was transferred to a well of microtiter plates in columns 3 – 10 (A – H). From the wells in column 10 (A – H), 125 μL of mixture was removed. The same procedure was repeated in the other two microtiter plates for the same natural substance.

Dilution range of the second compound was prepared in other microtiter plate in the same way with use of two-fold dilution. The entire concentration range of the second compound was 15 times more concentrated due to its transfer of 10 μL to wells of microtiter plate containing the first compounds. Finally, microbial suspension was added. All determinations of antimicrobial activity included the control of growth of microorganisms in MHB without inhibiting substances, without solvent and control of medium sterility. Concentration of ethanol did not exceed 2% after final preparation.

Analysis of activities of natural substances in combination

For determination of interaction of natural substances in combination, we modified MIC and MBC on MIC_{50} and MBC_{50} due to simplification. MIC and MBC values of natural compounds and EDTA combinations were determined together with the MIC and MBC values of single natural compounds and EDTA. The reason was the comparison of the results at the same time and under the same conditions, in terms of identifying interactions between substances. The types of interactions were analyzed based on the FICI (Fractional Inhibitory Concentration Index) and FBCI (Fractional Bactericidal Concentration Index) calculation by Hamoud *et al*. (2014) and Agboke *et* Esimone (2011). These four types of interactions were: synergy, additivity, indifference and antagonism.

Calculation of FICI and FBCI

$$\text{FICI for } MIC_{50} = FIC_A + FIC_B =$$

$$= \left(\frac{\text{MIC50 of compound A in combination}}{\text{MIC50 of single compound A}}\right) + \left(\frac{\text{MIC50 of compound B in combination}}{\text{MIC50 of single compound B}}\right)$$

$$\text{FBCI for } MBC_{50} = FBC_A + FBC_B =$$

$$= \left(\frac{\text{MBC50 of compound A in combination}}{\text{MBC50 of single compound A}}\right) + \left(\frac{\text{MBC50 of compound B in combination}}{\text{MBC50 of single compound B}}\right)$$

FIC_A – fractional inhibitory concentration of compound A

FIC_B – fractional inhibitory concentration of compound B

FBC_A – fractional bactericidal concentration of compound A

FBC_B – fractional bactericidal concentration of compound B

Evaluation of interactions of natural substances in combination - adopted and modified according to Hamoud *et al*. (2014)

FICI, FBCI ≤ 0,5; Synergistic interaction of natural substances in combination

FICI, FBCI > 0,5 ≤ 1; Additional interaction of natural substances in combination

FICI, FBCI > 1 ≤ 4; Indifferent interaction of natural substances in combination

FICI, FBCI > 4; Antagonistic interaction of natural substances in combination

2.6 METHOD FOR DETERMINATION OF SPERMIOTOXICITY OF NATURAL SUBSTANCES

Freshly collected boar semen was diluted with the Beltsville Thawing Solution (BTS) in a ratio of 1:3 in a test-tubes preheated to 37°C. Subsequently, 0.1 mL of calculated amount of natural substance dissolved in ethanol and physiologic saline was added to the test-tubes after they were slowly cooled to 17°C. After the gentle but thorough stirring of the mixture, sperm motility was observed by phase contrast microscopy. The specimen was prepared by adding 15 μL of the mixture to a microscope slide and covered with a cover slip, both preheated to 37°C to activate the sperm. Motility evaluation was performed 0, 1, 4, 24, 48 and 72 hours after the addition of natural substance. Maximum non-toxic concentration of natural substances was evaluated as the highest concentration of natural compound in which at least 10 % of motile sperm cells was observed.

3 RESULTS AND DISCUSSION

3.1 RESULTS OF QUALITATIVE AND QUANTITATIVE EXAMINATION OF BOAR SEMEN

All examined ejaculates collected from boars at the Animal Production Research Institute in Kostelec nad Orlicí had contained microorganisms. Their qualitative and quantitative representation is shown in Table 1. Most frequently, *Pseudomonas aeruginosa* and *Enterobacteriaceae* species were present.

Table 1: Microorganisms isolated from boar semen specimens (n = 30)

Microorganisms	Microorganisms in boar semen		Species representation according to quantities[1]		
	Absolutely	%	$10^1 – 10^2$	$10^3 – 10^4$	$>10^5$
Pseudomonas aeruginosa	17	56.7	4	8	5
Escherichia coli	6	20.0	2	3	1
Morganella morganii	1	3.3	1	0	0
Providencia stuartii	3	10.0	2	1	0
Proteus mirabilis	7	23.3	3	2	2
Klebsiella sp.	4	13.3	N	N	N
Gram-negative non-fermenting rods	5	16.7	4	1	0
Staphylococcus simulans	3	10.0	1	2	0
Staphylococcus hyicus	1	3.3	N	N	N
Staphylococcus haemolyticus	2	6.7	1	1	0
Other coagulase-negative staphylococci	3	10.0	3	0	0
Viridans streptococci	5	16.7	1	2	2
Beta-hemolytic streptococci	4	13.3	3	1	0
Enterococcus sp.	5	16.7	5	0	0
Yeasts	2	6.7	2	0	0
Coryneform rods	4	13.3		3	1
Others	6	20.0	N	N	N

N – not determined

[1] – Quantities (10^1-10^2, 10^3-10^4, > 10^5) are in CFU/mL

Bacteriological examination proved that among the most common bacteria contaminating boar semen were *Ps. aeruginosa*, *Enterobacteriaceae* species, coagulase-negative staphylococci, enterococci and streptococci. Our findings are consistent with the results

of Bresciani *et al*. (2014); Sone *et al*. (1989); Althouse *et al*. (2005). In particular *Ps. aeruginosa* is problematic due to natural resistance to many antibiotics and ability to multiply at lower temperatures (15 – 17°C) in which insemination doses are stored. This species significantly reduces the amount of sperm cells with progressive motility, vitality and acrosome integrity. It is a microorganism resistant to environmental conditions and rapidly spreading among boars in stocks (Bresciani *et al*., 2014; Sepúlveda *et al*., 2014).

3.2 ANTIMICROBIAL ACTIVITIES OF NATURAL SUBSTANCES ON MICROORGANISMS ISOLATED FROM BOAR SEMEN AND OTHER CLINICAL SPECIMENS

Table 2a: MIC and MBC values (μg.mL^{-1}) of selected natural substances dissolved in 1% ethanol for microorganisms isolated from boar semen determined at time intervals by the microdilution method

Tested compound		*Pseudomonas aeruginosa* (n = 17)		*Escherichia coli* (n = 6)		*Proteus mirabilis* (n = 7)		*Providencia stuartii* (n = 3)	
	T[1]	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Thymol	24	150 - 2400	300 - 2400	150 - 600	150 - 600	300	300	300	300
	48	300 - 2400	600 - 2400	150 - 600	150 - 600	300	300	300	300
	72	300 - 2400	600 - 2400	150 - 600	150 - 600	300	300	300	300
Carvacrol	24	150 - 1200	150 - 2400	75 - 300	75 - 300	300	300	600	600
	48	150 - 1200	150 - 2400	75 - 300	75 - 300	300	300	600	600
	72	150 - 1200	150 - 2400	75 - 300	75 - 300	300	300	600	600
Cnicin	24	300	300 - 600	300 - 600	300 - 600	600	600	150 - 300	300
	48	300 - 600	300 - 600	300 - 600	300 - 1200	600	600	300	300
	72	300 - 600	300 - 600	300 - 600	300 - 1200	600	600	300	300
Usnic acid	24	> 4800	> 4800	> 4800	> 4800	-[2]	-	1200	2400
	48	> 4800	> 4800	> 4800	> 4800	-	-	2400	> 4800
	72	> 4800	> 4800	> 4800	> 4800	-	-	2400	> 4800
Gallic acid	24	150 - 300	2400 - 4800	4800	4800	2400	2400	1200	4800
	48	150 - 300	2400 - 4800	4800	4800	2400	2400	1200	4800
	72	150 - 300	2400 - 4800	4800	4800	2400	2400	1200	4800
Octyl gallate	24	1200 - 4800	1200 - 4800	300	300	-	-	150	150
	48	1200 - 4800	1200 - 4800	600	600	-	-	150	150
	72	1200 - 4800	1200 - 4800	600	600	-	-	150	150

[1] *Time of evaluation* [2] *not performed*

Table 2b: MIC and MBC values (µg.ml-1) of selected natural substances dissolved in 1% ethanol for microorganisms isolated from boar semen and other clinical specimens determined at time intervals by the microdilution method

Tested compound		*Enterococcus* sp. (n = 20)		*Staphylococcus* sp. (n = 7)		Beta-hemolytic streptococci (n = 4)		*Klebsiella* sp. (n = 4)		*Candida catenulata* (n = 1)	
	T[1]	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Thymol	24	150 - 1200	150 - 1200	300 - 600	300 - 600	600	600	150	300	300	300
	48	150 - 1200	150 - 1200	300 - 600	300 - 600	600	600	300	300	300	300
	72	150 - 1200	150 - 1200	300 - 600	300 - 600	600	600	300	300	300	300
Carvacrol	24	75 - 600	75 - 600	300 - 600	300 - 600	300	300	2400	2400	75	75
	48	75 - 600	75 - 600	300 - 600	300 - 600	300	300	2400	2400	150	150
	72	75 - 600	75 - 600	300 - 600	300 - 600	300	300	2400	2400	150	150
cnicin	24	600 - 1200	600-4800	-[2]	-	300	300	1200	1200	1200	1200
	48	600 - 1200	1200-4800	-	-	300	300	1200	1200	1200	1200
	72	600 - 1200	1200-4800	-	-	300	300	1200	1200	1200	1200
Usnic acid	24	4,7 - 37,5	-	9,4	-	4,7	-	> 9600	> 9600	> 9600	> 9600
	48	9,4 - 37,5	-	9,4	-	9,4	-	> 9600	> 9600	> 9600	> 9600
	72	9,4 - 37,5	-	9,4	-	9,4	-	> 9600	> 9600	> 9600	> 9600
Gallic acid	24	> 4800	> 4800	2400 - 4800	4800	4800	4800	4800	4800	4800	> 4800
	48	> 4800	> 4800	2400 - 4800	4800	4800	4800	4800	4800	> 4800	> 4800
	72	> 4800	> 4800	2400 - 4800	4800	4800	4800	4800	4800	> 4800	> 4800
Octyl gallate	24	37,5 - 75	37,5 - 75	-	-	18,8	18,8	-	-	4,7	4,7
	48	75 - 150	75 - 150	-	-	18,8	18,8	-	-	4,7	4,7
	72	75 - 150	75 - 150	-	-	18,8	18,8	-	-	4,7	4,7

[1] *Time of evaluation* [2] *not performed*

To determine the antimicrobial effects of natural substances we used 3 procedures - microdilution method, dilution method and agar dilution method. For further testing, microdilution method was selected due to good reproducibility, lower requirements for work time and better cost effectivity. MIC and MBC values of selected natural substances determined by the microdilution method after 24, 48 and 72 hours of incubation are in Tables 2a – b. For most compounds, we did not find any significant differences between 24 and 48 hour incubation. The MIC and MBC values after 72 hours were almost the same as those after 48 hours of incubation.

From the results shown in Tables 2a – b, it is obvious that the most effective against microorganisms were thymol and carvacrol. Both compounds can be considered as antimicrobial substances with a wide spectrum of efficacy. Thymol MIC values for strains *Ps. aeruginosa* (n = 17) were in the range of 300 – 2400 $\mu g.mL^{-1}$. Similar results were published by Walsh *et al*. (2003), which also used ethanol as solvent. Those authors demonstrated MIC thymol for *Ps. aeruginosa* 1000 $\mu g.mL^{-1}$ as well as Soković *et al*. (2010). In our study, growth of enterococci was inhibited by thymol in concentration range of 300 – 1200 $\mu g.mL^{-1}$. Similar results were published by Gutiérrez-Fernández *et al*. (2013).

During the testing of the antimicrobial effects of carvacrol against *Ps. aeruginosa* strains, we have shown that the compound inhibited this bacterium at concentrations of 150 – 1200 $\mu g.mL^{-1}$. The range of MBC values of carvacrol was 150 – 2400 $\mu g.mL^{-1}$. Similar results were achieved by Soković *et al*. (2010) who reported the MIC value of 500 $\mu g.mL^{-1}$ for *Ps. aeruginosa*. MIC values of carvacrol for enterococci species were in the range of 300 – 600 $\mu g.mL^{-1}$ excluding *E. faecium* strains with the range of MIC values was 75 – 150 $\mu g.mL^{-1}$. Similar results (MIC 600 – 800 $\mu g.mL^{-1}$) for *E. facalis* were reported by Gutiérrez-Fernández *et al*. (2013).

Eugenol is the main compound presented in cloves. It was previously known for his anti-inflammatory and analgesic effects. Its antibacterial effects were published by Dorman *et* Deans, (2000) or Hemaiswarya *et* Doble, (2009). Antibacterial activity of eugenol is lower compared to thymol and carvacrol as we confirmed in our work (see Tables 2a – b). For *Ps. aeruginosa* strains we determined the MIC of eugenol 1200 – 4800 $\mu g.mL^{-1}$, for enterococci the MIC ranged between 1200 – 2400 $\mu g.mL^{-1}$, the MBC values were the same. According to Medina *et al*. (2009) and Joshi (2013) eugenol inhibited *Ps. aeruginosa* in the range of 1200 – 2400 $\mu g.mL^{-1}$, which is consistent with our results. Significantly lower MIC values of 273 $\mu g.mL^{-1}$ and MIC 200 $\mu g.mL^{-1}$ were found by Bassolé *et al*. (2010) for *E. faecalis*.

Gallic acid was much less effective on enterococci than on *Ps. aeruginosa* (see Table 2a – b). Our findings are confirmed by studies of Barcelo *et al*. (2014) who reported the MIC values of gallic acid for *E. faecalis* $62.5x10^3$ $\mu g.mL^{-1}$ and *Ps. aeruginosa* 500 $\mu g.mL^{-1}$. The MIC values of gallic acid determined in our work are for most of the *Ps. aeruginosa* strains in range of 150 – 300 $\mu g.mL^{-1}$, MBCs were significantly higher (2400 – 4800 $\mu g.mL^{-1}$). However, we consider gallic acid as a substance effective against for *Ps. aeruginosa*.

In case of octyl gallate, pronounced antifungal, antibacterial and antiviral activities were proved (Kubo *et al*., 2002; Ha *et al*., 2004; Gutiérrez-Larraínzar *et al*., 2013). We also confirmed significant activities of octyl gallate against gram-positive aerobic or anaerobic bacteria and yeasts. MIC values of *St. aureus, Candida albicans, Clostridium perfringens* and *Bacteroides fragilis* were 18.8 $\mu g.mL^{-1}$. Our results are similar to Kubo *et al*. (2001), authors reported octyl gallate MIC of 25 $\mu g.mL^{-1}$ for *St. aureus* a *Candida albicans*.

Another compound – usnic acid is naturally presented in lichen of genus *Parmelia* or *Usnea*.

In our study, usnic acid had the strongest inhibitory effects on gram-positive bacteria with range of MIC values of 4.7 – 37.5 $\mu g.mL^{-1}$ (see Table 2b). Our results are similar to those reported by Lauterwein *et al*. (1995). Usnic acid is not effective against gram-negative bacteria and yeasts.

In our work we tested cnicin extracted from *Cnicus benedictus* seeds by column chromatography. It is a substance with relatively universal effects on gram-positive and gram negative bacteria. MIC values ranged among 300 – 600 $\mu g.mL^{-1}$ with the exception of some enterococci, *Klebsiella pneumoniae* and yeasts. Unlike other tested substances, cnicin has a specific mechanism of action on bacteria consisting of inhibiting the initial step of peptidoglycan biosynthesis, and thus biosynthesis of bacteriacell wall is stopped (Bugg *et al*., 2011). So far, there is little evidence in the literature about the antibacterial effects of cnicin that would be comparable with our results. Bruno *et al*. (2003) reported MIC values for *St. aureus, Proteus mirabilis, Ps. aeruginosa* a *E. coli* 25, 25, 50 and 12.5 $\mu g.mL^{-1}$, respectively. In study of Mazurová *et al*. (2007), MIC values of cnicin dissolved in ethanol for *E. faecalis* were in range of 600 – 2400 $\mu g.mL^{-1}$ and 300 – 600 $\mu g.mL^{-1}$ for *Ps. aeruginosa*.

The mechanism of the most natural compounds action excluding cnicin is based mainly on their ability to disrupt the integrity of the microbial cytoplasmic membrane and to interfere with the metabolism of bacteria. The disruption of membrane leads to leakage important intracellular compounds and ions resulting in possible death of microbial cell (Kubo *et al*., 2004; Kubo *et al*., 2010; Rangel *et al*., 2010).

3.3 ANTIMICROBIAL ACTIVITIES OF NATURAL SUBSTANCES AND EDTA IN DOUBLE COMBINATIONS

The objective of verifying the antimicrobial effects of natural substances in double combinations was also to investigate mutual interactions. We analyzed whether a synergistic effect occurs when the FICI/FBCI is ≤ 0.5; additive effect (FICI/FBCI > 0.5 ≤ 1), indifference (FICI/FBCI > 1 ≤ 4) or antagonistic effects (FICI/FBCI > 4). In this annotation, we mention only the most effective double combinations of natural substances.

Strains of *E. faecalis*, *E. faecium* and *Ps. aeruginosa* were strongly inhibited by combinations of phenolic compounds, especially carvacrol or thymol with EDTA. Effective concentrations of thymol or carvacrol in mixture with EDTA were several times lower compared to MIC_{50} and MBC_{50} alone, indicating synergistic effects (see Table 3). We have confirmed these findings by calculating the FICI and FBCI by Hamoud *et al*. (2014). It was a combination of thymol with EDTA on strains *Ps. aeruginosa*, when comparing the MIC_{50} and MBC_{50} values of individual substances with MIC_{50} and MBC_{50} of combination of these substances. We calculated the FICI 0.31 and 0.19, respectively, meaning synergistic effect. For combination of thymol and EDTA against *E. faecalis* and *E. faecium* strains, we found additive effects.

Combination of carvacrol with EDTA was effective against *E. faecalis* and *Ps. aeruginosa* strains, showing also synergistic effects. Similar results were reported by Hamoud *et al*. (2014). The synergistic effects of carvacrol with antibiotics have been demonstrated in study by Fadli *et al*. (2012)

The additive and synergistic effects of the combination of EDTA with thymol or carvacrol may be elucidated by the ability of EDTA to reduce the resistance of the bacterial cell wall and allow the penetration of natural substances into the cytoplasmic membrane and into the bacterial cell Hamoud *et al*. (2014).

Table 3: Example of the MIC_{50} and MBC_{50} values ($\mu g.mL^{-1}$) of natural substances combination determined by the microdilution method and analysis of their interactions for microorganisms isolated from boar semen and other clinical specimens

		Thymol	EDTA	Thymol+EDTA	FICI/*FBCI*	Effect
Enterococcus faecalis (n = 6)	MIC_{50}	600	600	300+18,8	0,53	ADI
	MBC_{50}	600	1200	300+37,5	*0,53*	ADI
Enterococcus faecium (n = 6)	MIC_{50}	600	300	150+75	**0,50**	**SYN**
	MBC_{50}	600	600	75+300	*0,63*	ADI
Pseudomonas aeruginosa (n = 10)	MIC_{50}	600	1200	150+75	**0,31**	**SYN**
	MBC_{50}	1200	1200	150+75	***0,19***	**SYN**

		Thymol	Methyl gallate	Thymol+methyl gallate	FICI/*FBCI*	Effect
Enterococcus faecalis (n = 6)	MIC_{50}	600	2400	300+300	0,63	ADI
	MBC_{50}	600	2400	-[1]	-	-
Pseudomonas aeruginosa (n = 10)	MIC_{50}	600	1200	300+300	0,75	ADI
	MBC_{50}	1200	1200	600+600	*1,00*	ADI

		Thymol	Ethyl gallate	Thymol+ethyl gallate	FICI/*FBCI*	Effect
Enterococcus faecalis (n = 6)	MIC_{50}	600	2400	300+300	0,63	ADI
	MBC_{50}	600	2400	600+600	*1,25*	IND
Pseudomonas aeruginosa (n = 10)	MIC_{50}	600	1200	600+600	1,5	IND
	MBC_{50}	1200	2400	600+600	*0,75*	ADI

		Thymol	Carvacrol	Thymol+carvacrol	FICI/*FBCI*	Effect
Enterococcus faecalis (n = 6)	MIC_{50}	600	300	75+75	**0,38**	**SYN**
	MBC_{50}	600	600	150+150	***0,5***	**SYN**
Pseudomonas aeruginosa (n = 10)	MIC_{50}	600	600	300+600	1,5	IND
	MBC_{50}	1200	1200	300+600	*0,75*	ADI

FICI/*FBCI: Fractional Inhibitory/Bactericidal Concentration Index*

MIC_{50}: Minimum Inhibitory Concentration for 50% of bacterial cells

MBC_{50}: Minimum Bactericidal Concentration for 50% of bacterial cells

SYN (synergistic effect): FICI ≤ 0,5 *ADI (additive effect): FICI > 0,5 to ≤ 1*

IND (indiferent effect): FICI > 1 to ≤ 4 *ANT (antagonistic effect): FICI > 4*

[1] *not evaluated*

3.4 SPERMIOTOXICITY OF NATURAL SUBSTANCES FOR BOAR SPERM CELLS

The method is difficult to perform, because boar sperm, unlike other mammalian sperm, are very sensitive to heat shock, oxidative damage of the cytoplasmic membrane for high concentration of unsaturated fatty acids, and relatively low antioxidant capacity of the seminal plasma (Přinosilová *et al*., 2012).

Results were expressed as a percentage of the motile sperm cells in boar semen observing in the phase microscope at different time intervals from the addition of different concentrations of the natural substance. Examples of results for thymol, carvacrol, EDTA and control are in Figures 5 – 7. The maximum non-toxic concentration was defined as the highest concentration of natural substance in which at least 10% of motile sperm cells was observed in the sample.

The lowest toxicities were determined for EDTA, carvacrol and thymol. Carvacrol and thymol are the most effective compounds tested in our work, suggesting their possible use in boar semen decontamination. Other natural substances with low toxicity for boar sperm cells but also lower antimicrobial activity were gallic acid, eugenol, methyl gallate and ethyl gallate (data not shown). To our knowledge, there is no information in available studies about determination of natural substances toxicity for boar sperm cell.

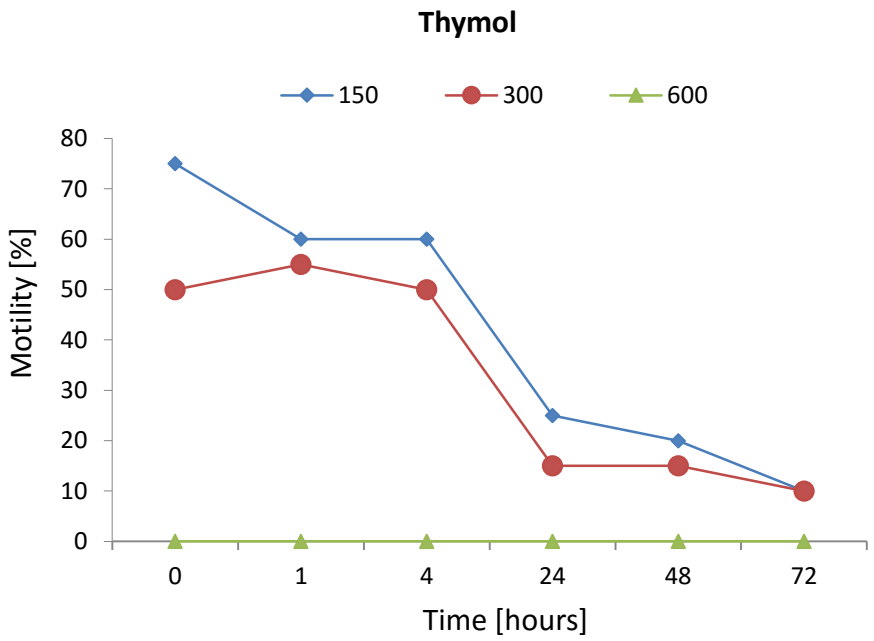

Figure 5: Expression of boar sperm cells motility in different concentrations (μg.mL^{-1}) of thymol in time intervals

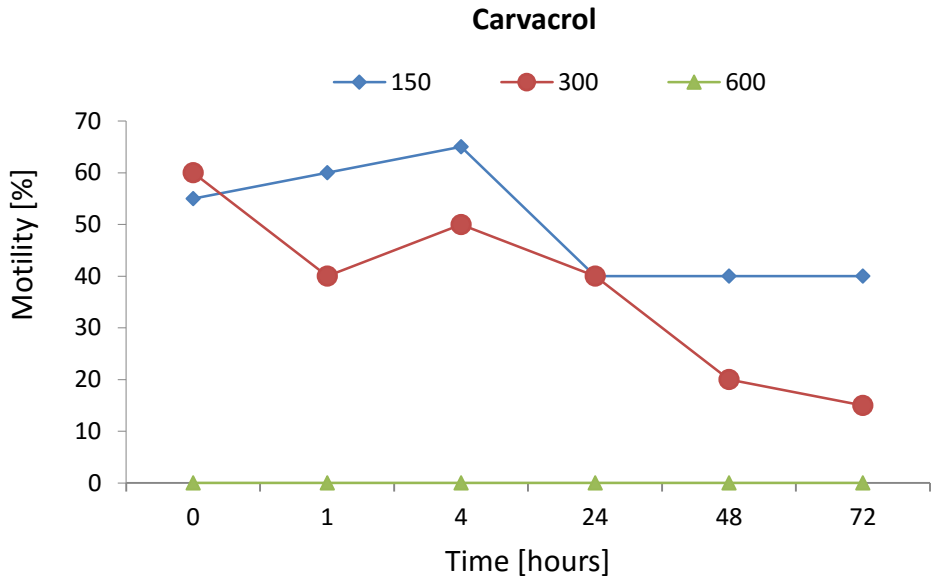

Figure 6: Expression of boar sperm cells motility in different concentrations (μg.mL^{-1}) of carvacrol in time intervals

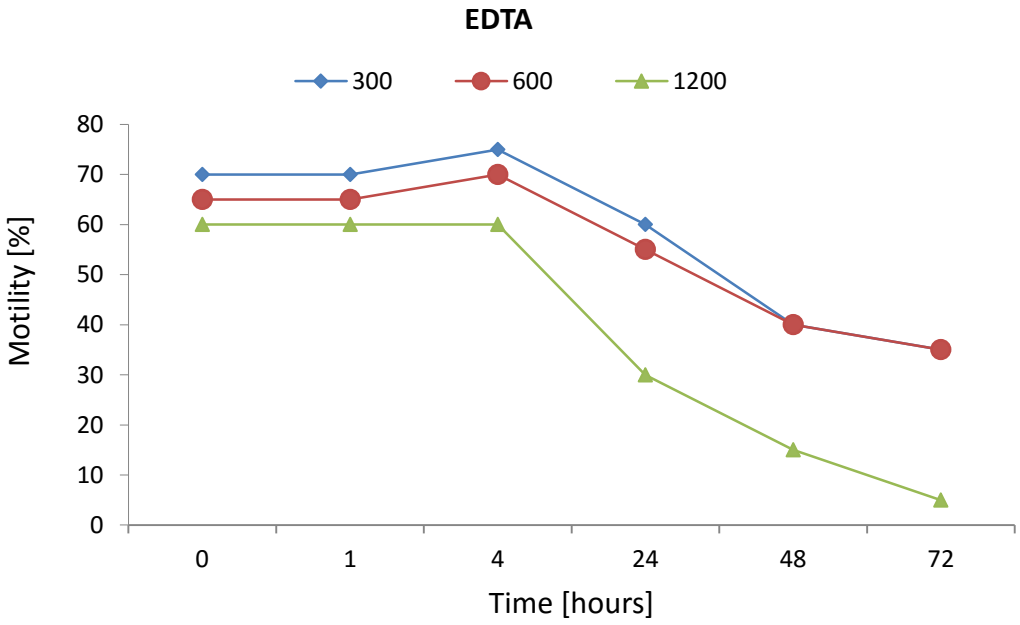

Figure 7: Expression of boar sperm cells motility in different concentrations (μg.mL^{-1}) of EDTA in time intervals

4 CONCLUSION

The study was focused on the introduction of methods for verifying the antimicrobial activity and spermiotoxicity of natural substances and essential oils aiming on their possible use for the boar semen decontamination. To determine the antimicrobial effects of natural substances and their combinations, we have selected the microdilution method. On the basis of the literature and our preliminary results, we continued with testing of 10 compounds (thymol, carvacrol, eugenol, gallic acid, methyl gallate, ethyl gallate, propyl gallate, octyl gallate, knicin, usnic acid) and EDTA. For these substances, we determined the MIC and MBC values.

To determine the antibacterial effects of natural substances and EDTA combination, we compared MIC_{50} and MBC_{50} with the combination of test substances with MIC_{50} and MBC_{50} values for individual substances. The mutual effects of natural substances combination (synergistic, additive, indifferent and antagonistic) were analyzed according to FICI and FBCI values. The most effective combination against enterococci and *Ps. aeruginosa* strains were carvacrol with EDTA (FICI = 0.19 – 1.25), thymol with EDTA (FICI = 0.19 – 0.63) and carvacrol with thymol (FICI = 0.38 – 1.5). The mutual interactions of these double combinations were mostly evaluated as synergic or additive.

Determination of antimicrobial activity of natural substances and their toxicity for boar sperm cells revealed thymol, carvacrol and EDTA as possible agents for boar semen decontamination. Other compounds active against microorganisms (usnic acid, octyl gallate, cnicin) were toxic for boar sperm cells. Nevertheless, these substances may be used for further research in human or veterinary medicine.

In verifying the toxic effects of natural substances, we have shown that only thymol, carvacrol or EDTA and mainly their double combinations are less toxic for boar sperm cells at concentrations effective on microorganisms than the other tested compounds. These compounds and their combinations may be potentially used for decontamination of boar insemination doses. This assumption, however, needs to be verified on higher number of boar semen specimens. The importance in searching for new sources of antimicrobial substances is obvious due to excessive use of antibiotics and increasing resistance of microbes against antibiotics. Natural substances are considered to be promising antimicrobial compounds having unspecific mechanisms of action; therefore development of specific resistance is rare.

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Results of the thesis had been presented in 3 lectures and 2 posters at international conferences and 1 lecture at national conference by the author since 2011 to 2015.

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Web links for substances structures

[1] http://www.chemspider.com/Chemical-Structure.21105998.html

[2] http://www.chemspider.com/Chemical-Structure.21105867.html

[3] https://pubchem.ncbi.nlm.nih.gov/ compound/Octyl_gallate#section=Top

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