

BACTERICIDAL ACTIVITY OF NATURAL SUBSTANCES ON MICROORGANISMS CONTAMINATING BOAR SEMEN

J. Mazurova, P. Lyskova, M. Vydrzalova, M. Capkova, T. Kroupa

Department of Biology and Biochemistry, Faculty of Chemical Technology, University of Pardubice

Introduction

Microorganisms contaminating boar semen are one of the most important factors that negatively affect the biological quality of spermatozoa. *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus* sp., staphylococci and enterococci belong to the most frequently isolated bacterial species. Their strong biochemical activity leads to the decrease in energetic sources of seminal plasma and production of metabolites toxic to reproductive cells. Various antimicrobial agents are used to suppress the growth of microorganisms in boar ejaculates. However, long-term use of these drugs causes bacterial resistance, which is a growing health problem in general.

The aim of our study was to determine antibacterial activity of various natural substances against microorganisms contaminating boar raw ejaculates.

Material and methods

Minimal bactericidal concentrations (MBC) of 20 natural substances extended in 5% dimethyl sulfoxide (DMSO) and 96% ethanol were determined by broth dilution and microdilution method in Mueller-Hinton broth.

Staphylococcus aureus CCM 3953, *Escherichia coli* CCM 3954, *Enterococcus faecalis* CCM 4224 and *Pseudomonas aeruginosa* CCM 3955 reference strains, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus hyicus* strains isolated from boar ejaculates and one *Pseudomonas aeruginosa* strain isolated from external ear canal of dog with otitis externa were the bacterial strains tested. The bacterial inoculum of each strain was prepared at the concentration of $7,5 \cdot 10^6$ /ml from fresh colonies grown on blood agar.

Dilutions tested ranged from 18,8 to 38400 µg/ml for all substances and were prepared using two-fold dilution procedure. Each tube was inoculated with 100 µl of the standardized, diluted bacterial suspension at the concentration of $7,5 \cdot 10^6$ /ml and incubated at 37°C for 72 h.

Results were analyzed visually every 24 hours and classified according to the following patterns: turbidity with bacterial colonies being deposited and total growth inhibition. Substances that showed inhibitory activity were submitted to a subculture of the broth media on blood agar in order to evaluate bacterial growth. The concentration at which there was no bacterial growth after inoculation on blood agar was taken as the minimal bactericidal concentration.

Results and discussion

MBCs of all natural substances tested are shown in table 1. Apparently, the results differed depending on the used diluent. Some of the substances prepared in 5% DMSO solution didn't dissolved completely and formed suspension (cnicin) or emulsion (thymol). To solve this problem we diluted some of the substances also in 96% ethanol. Furthermore, our study proved wide interspecies variation in susceptibility to natural substances. *Pseudomonas aeruginosa* (G-negative bacteria) and *Enterococcus faecalis* (G-positive bacteria) were found to be the least susceptible of all bacterial species tested.

Pseudomonas aeruginosa is commonly known for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen. However, cnicin, carvacrol and thymol exerted good effects on this bacterium. MBCs of substances in 96% ethanol ranged from 150-600 µg/ml and MBCs of substances in 5% DMSO solution ranged from 600-2400 µg/ml. Also enterococci belong to microorganisms resistant to many commonly used antimicrobial agents (see table 2). In our study, thymol and carvacrol extended in 96% ethanol (MBC 150-600 µg/ml) were the most effective substances used against *Enterococcus faecalis* (see table 3). In conclusion, carvacrol (300-600 µg/ml), cnicin (600-2400 µg/ml), thymol (150-600 µg/ml) and eugenol (600-4800 µg/ml) extended in 96% ethanol were the most effective natural extracts against all bacterial species included in our study.

Future

Effects of natural substances on microorganisms contaminating real specimens of boar ejaculates is going to be object of further studies of our laboratory.

Table 1 Antibacterial activity of natural substances on all microorganisms included in our study determined by dilution methods

Natural substance	Minimal bactericidal concentration ($\mu\text{g/ml}$) determined by:		
	broth dilution method		broth microdilution method
	extended in 5% DMSO	extended in 96% ethanol	extended in 96% ethanol
Arbutin	>38400 ^a	9600-19200 ^a	N
Carvacrol	600-2400 ^c	300-600 ^a	300-2400 ^a
Cnicin	2400->19200 ^c	600-2400 ^a	300-1200 ^a
p-cymen	>38400 ^b	19200-38400 ^a	>38400 ^a
Eugenol	600-9600 ^b	600-4800 ^a	600-9600 ^a
Ethylgallate	2400->4800 ^a	2400-9600 ^a	600-2400 ^a
Methylgallate	4800->9600 ^a	2400-9600 ^a	300-2400 ^a
Propylgallate	N	2400-9600 ^a	N
Gallic acid	19200->38400 ^c	2400-9600 ^a	N
Hydroquinone monomethylether	>4800 ^a	1200-4800 ^a	N
Rosmarinic acid	19200->38400 ^c	4800-9600 ^a	N
Thymol	300-2400 ^b	150-600 ^a	150-2400 ^a
Quercetin dihydrate	>19200 ^c	9600-19200 ^a	N
Carvi etheroleum	> 9200 ^b	2400-9600 ^a	N
Caryophilli etheroleum	1200-19200 ^b	600-9600 ^a	N
Eucalypti etheroleum	>19200 ^b	4800-9600 ^a	N
Foeniculi etheroleum	9600->19200 ^b	4800-19200 ^a	N
Lavandulae etheroleum	>19200 ^b	4800-19200 ^a	N
Rosmarini etheroleum	19200->38400 ^b	4800-9600 ^a	N
Tea tree oil	>38400 ^b	4800-19200 ^a	N

N Not tested. ^a Solution. ^b Emulsion. ^c Suspension.

Table 2 Minimal bactericidal concentration of natural substances on *Pseudomonas aeruginosa* strains determined by dilution methods

Natural substance	Minimal bactericidal concentration ($\mu\text{g/ml}$) determined by:		
	broth dilution method		broth microdilution method
	extended in 5% DMSO	extended in 96% ethanol	extended in 96% ethanol
Arbutin	>38400	9600	N
Carvacrol	2400	300-600	600-2400
Cnicin	2400-9600	300-600	300
p-cymen	>38400	19200	38400
Eugenol	9600	600-4800	600-9600
Ethylgallate	4800	1200-2400	600-1200
Methylgallate	4800-9600	2400	600-2400
Propylgallate	N	2400	N
Gallic acid	19200-38400	2400-4800	N
Hydroquinone monomethylether	4800	1200	N
Rosmarinic acid	38400	4800-19200	N
Thymol	600-2400	150-600	600-2400
Quercetin dihydrate	>19200	9600	N
Carvi etheroleum	>19200	9600	N
Caryophilli etheroleum	9600-19200	4800-9600	N
Eucalypti etheroleum	>19200	9600	N
Foeniculi etheroleum	>19200	9600-19200	N
Lavandulae etheroleum	>19200	19200	N
Rosmarini etheroleum	>38400	4800-9600	N
Tea tree oil	>38400	9600-19200	N

N Not tested.

Table 3 Minimal bactericidal concentration of natural substances on *Enterococcus faecalis* strains determined by dilution methods

Natural substance	Minimal bactericidal concentration ($\mu\text{g/ml}$) determined by:		
	broth dilution method		broth microdilution method
	extended in 5% DMSO	extended in 96% ethanol	extended in 96% ethanol
Arbutin	>38400	19200	N
Carvacrol	1200	300-600	300-1200
Cnicin	19200	600-2400	4800
p-cymen	>38400	38400	38400
Eugenol	>4800	1200-2400	1200-4800
Ethylgallate	>9600	9600	2400
Methylgallate	4800-9600	2400	2400
Propylgallate	N	4800-9600	N
Gallic acid	>38400	9600	N
Hydroquinone monomethylether	>4800	4800	N
Rosmarinic acid	19200	4800-9600	N
Thymol	600-2400	150-600	300-600
Quercetin dihydrate	>19200	19200	N
Carvi etheroleum	>19200	4800	N
Caryophilli etheroleum	2400-9600	2400	N
Eucalypti etheroleum	19200	9600	N
Foeniculi etheroleum	>19200	4800	N
Lavandulae etheroleum	19200	19200	N
Rosmarini etheroleum	38400	4800-9600	N
Tea tree oil	>38400	4800-9600	N

N Not tested

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