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Use of natural substances for boar semen decontamination

J. Mazurova[1], R. Kukla[1], M. Rozkot[2], A. Lustykova[2], E. Slehova[1], R. Sleha[1,3], J. Lipensky[2], L. Opletal[4]

[1]Faculty of Chemical-Technology, University of Pardubice, Czech Republic
[2]Institute of Animal Science, Prague-Uhrineves, Workplace Kostelec nad Orlici, Czech Republic
[3]Faculty of Military Health Sciences, University of Defence, Hradec Kralove
[4]Faculty of Pharmacy, Hradec Kralove, Charles University, Prague, Czech Republic

ABSTRACT: The aim of this study was to investigate the antibacterial activity and toxicity for sperm cells of the natural substances gallic acid, methyl gallate, ethyl gallate, propyl gallate, octyl gallate, thymol, carvacrol and eugenol. The antibacterial activity of these natural substances and selected combinations of them against bacterial strains isolated from boar ejaculates was determined using the microdilution and macrodilution method in Mueller-Hinton broth. The most effective natural substances against Gram-negative and Gram-positive bacteria included in our study were thymol and carvacrol with minimum inhibitory concentration (MIC) values in the range of 300–600 µg/ml. Gallic acid exhibited the best antibacterial activity against *Pseudomonas aeruginosa* strains (MIC values of 300–2400 µg/ml), whereas the ranges of MIC values against *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus* sp. strains were higher. Octyl gallate exhibited stronger antibacterial activity against staphylococci and enterococci (MIC values of 18.8–75 µg/ml) than against *Escherichia coli* and *Pseudomonas aeruginosa* strains with MIC values in the ranges of 300–600 µg/ml and 1200–2400 µg/ml, respectively. Thymol combined with carvacrol was the most effective combination against enterococci (MIC values of 75–300 : 150 thymol : carvacrol) and *Pseudomonas aeruginosa* (MIC values of 75–300 : 300 thymol : carvacrol), bacteria which are known to be frequently resistant to antimicrobials. Similar results were determined for the combination of carvacrol and eugenol against staphylococci and enterococci. The results of the combinations revealed more of an additive rather than a synergistic effect. Thymol and carvacrol were the most effective natural substances against the bacteria included in this study, with a low toxicity for sperm cells compared to other substances, suggesting their possible use for boar semen decontamination.

Keywords: thymol; carvacrol; eugenol; gallic acid; gallates; antibacterial activity; bacteria; spermiotoxicity

A successful artificial insemination procedure may be adversely influenced for a range of reasons, including the contamination of ejaculates by various genera of microorganisms at various concentration levels. The majority of microorganisms found in native boar semen are species from the family *Enterobacteriaceae*, especially the genera *Eschericha*, *Enterobacter* and *Proteus*. Additionally, enterococci, staphylococci and *Pseudomonas aeruginosa* (*P. aeruginosa*) can be found. The majority of these bacteria are opportunistic metabolic active pathogens, which are able to decrease the biological quality of boar semen. Moreover, these microorganisms may participate in the induction of inflammation processes in the uterine mucosa of inseminated sows. In order to eliminate unfavourable bacteria, various antibiotics or combinations of them are added to semen extenders. Due to the increase in the resistance of microorganisms as a result of the global use or even overuse of antimicrobial agents not merely for therapeutic purposes, there is an effort to decrease antibiotic consump-

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tion or to replace antimicrobial agents. One of the possible alternatives may be the utilisation of natural substances with antimicrobial properties. These substances include gallic acid, methyl gallate, ethyl gallate, propyl gallate, octyl gallate, thymol, carvacrol and eugenol.

Gallic acid is a naturally occurring phenolic compound with antioxidant, antibacterial and antifungal activity. In contrast to the compounds mentioned below, gallic acid is highly soluble in water and in polar solvents such as ethanol or methanol (Daneshfar et al. 2008). This compound is found in hornbeam and oak bark, oak apple, green or black tea, hops, pomegranate and in other plants and fruits. Gallic acid may occur either as a free (unbound) molecule or it can be conjugated in molecules of tannins, from which it is separated by acid or thermal hydrolysis (Kim et al. 2011). In tannins, gallic acid forms esters with carbohydrates, especially with glucose. Gallate is a general term for salts and esters of gallic acid with the galloyl group (Takai et al. 2011). Methyl, ethyl, propyl, octyl and dodecyl gallate are considered to be important esters of gallic acid (Ivanova et al. 2002).

The mechanism of the antimicrobial action of phenolic compounds including gallic acid is based mainly on their ability to disrupt the integrity of the bacterial cytoplasmic membrane and to interfere with the metabolism of bacteria. Gallic acid also may create insoluble complexes with proteins or with Fe, Zn and Ca ions. Moreover, an ability to induce apoptosis was reported in studies by Strlic et al. (2002) and Ow and Stupans (2003).

Alkyl gallates are generally considered to be antioxidants, although bacteriostatic and bactericidal effects of gallates were established as early as 1953 in a study by Johnstone and Little (1953) who reported the inhibition of *Mycobacterium tuberculosis* metabolism by ethyl gallate and propyl gallate.

Methyl gallate is a major component of Galla Rhois, it also occurs in the leaves of *Sapium sebiferum* Roxb. (Kane et al. 1988; Choi et al. 2009). This substance exhibits antimicrobial activity against cariogenic bacteria including actinomycetes, streptococci, lactobacilli and against *Escherichia coli* (*E. coli*), *Salmonella* sp. and several other bacteria of the family *Enterobacteriaceae* (Choi et al. 2009). Moreover, the synergistic activity of methyl gallate with ciprofloxacin or nalidixic acid against these enterobacteria has been previously demonstrated (Choi et al. 2008; Choi et al. 2009). Kane et al. (1988) also reported that methyl gallate acts as specific inhibitor of herpes simplex virus (type 2) *in vitro*.

Ethyl gallate is slightly soluble in water but freely soluble in ether and ethanol. This compound is found in *Paeonia peregrina* Mill. and *Paeonia tenuifolia* L. roots. Zhou et al. (2007) determined a weak inhibitory activity of ethyl gallate against *Bacillus subtilis* (MIC value 1000 μg/ml). Shibata et al. (2005) reported that ethyl gallate could intensify beta-lactam susceptibility in methicillin-resistant and methicillin-sensitive strains of *Staphylococcus aureus* (*S. aureus*).

Propyl gallate, a propyl ester of gallic acid is partially soluble in water (Jacobsen et al. 1999) and freely soluble in common solvents such as *N*,*N*-dimethylformamide (Kubo et al. 2002), methanol (Sharma and Bhat 2009), ethanol and ether (Solorzano-Santos and Miranda-Novales 2012). This substance is known to exhibit antioxidant activity, but antibacterial activities were also reported (Kubo et al. 2001; Sharma and Bhat 2009).

Octyl gallate is an ester of the ubiquitously occurring natural substance gallic acid. It is almost insoluble in water, much less than the gallates mentioned above (Lu et al. 2007), while it is freely soluble in ethanol (Hsu et al. 2007) and methanol (Perrin and Meyer 2002). This compound is primarily known for its pronounced antioxidant properties (Ha et al. 2004). Nevertheless, octyl gallate is known to possess inhibitory activity against various species of fungi and bacteria, in particular against Gram-positive bacteria (Kubo et al. 2001; Rua et al. 2011). Rua et al. (2011) found a mean MIC value of 20.89 μg/ml for octyl gallate against *S. aureus* strains isolated from dairy and meat products. In addition, an antiviral effect against DNA as well as RNA viruses was established (Uozaki et al. 2007). The mechanism of antimicrobial action is associated with a balance between the hydrophobicity of the side chain and the hydrophobicity of hydroxyls on the benzene ring. Octyl gallate acts as non-ionic surfactant and is able to the inhibit efflux pumps of certain microorganisms (Kubo et al. 2004; Kubo et al. 2010; Rangel et al. 2010).

Thymol and carvacrol are well known phenolic isomeric monoterpenes found in mixtures with other substances in *Thymus vulgaris* L., *Origanum vulgare* L., *Satureja hortensis* L. and *Thymus pectinatus* L. Fisch. et Mey. var. *pectinatus* (Alma et al. 2003; Vardar-Unlu et al. 2003; Goren et al. 2004; Bendahoua et al. 2008). These compounds pos-

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sess a broad spectrum of antimicrobial activities against bacteria, yeasts and fungi (Garcia-Garcia et al. 2011; Darvishi et al. 2013). Depending on the concentration, thymol and carvacrol may exhibit either bacteriostatic or bactericidal activity. Their mechanism of action is most likely due to the disruption of the bacterial cytoplasmic membrane through the binding of the polysaccharide fraction to lipids, which increases the permeability and depolarises the potential of the membrane. This leads to increased permeability and to a leakage of ions and important nutrients and potentially, to bacterial cell death (Helander et al. 1998; Lambert et al. 2001; Hanbali et al. 2005; Trombetta et al. 2005; Cristani et al. 2007; Demirci et al. 2007).

Thymol is a white crystalline substance with a sharp odour resembling camphor, weakly soluble in water, where it forms an emulsion. It is highly soluble in ethanol, dilute hydroxide solutions, ether, paraffin oil and glycerol. At higher concentrations, thymol may evoke skin and mucosal irritation (Elissondo et al. 2008; Lee et al. 2008; Bassole et al. 2010; Archana et al. 2011). Besides damaging the bacterial cytoplasmic membrane with the subsequent death of bacteria, damage to the bacterial cell wall and the inhibition of some bacterial enzymes has also been reported (Farag et al. 1989). The antioxidant capacity of thymol has also been established (Vondruskova et al. 2010; Brewer 2011). Carvacrol, a regioisomer of thymol, is a brown viscous liquid, almost insoluble in water, where it forms an emulsion, soluble in ethanol, ether and common solvents. Its mechanism of action is similar to thymol; nevertheless, its MIC values may be different.

Eugenol is a phenylpropane derivative exhibiting pronounced antimicrobial, antioxidant and analgesic effects. Eugenol is a common constituent of *Eugenia caryophyllata* L. Merr. & Perry, *Myristica fragrans* Houtt., *Laurus nobilis* L., *Cinnamomum zeylanicum* L. and many other plants (Miller et al. 2000; Bassole et al. 2010). It is a yellowish liquid with a clove-like odour which is weakly soluble in water and highly soluble in organic solvents. The mechanism of its antimicrobial action may be explained by its effects on the function of the bacterial outer membrane and by its disruption of bacterial metabolism and proteosynthesis (Kalemba and Kunicka 2003; Cristani et al. 2007; Xu et al. 2008; Oyedemi et al. 2009; Devi et al. 2010; Silva and Fernandes 2010; Hyldgaard et al. 2012).

It is desirable to examine combinations of natural substances in order to determine whether they act synergistically or not. Synergistic combinations mean that minimum inhibitory concentrations are decreased. The antibacterial properties of combined natural substances were evaluated in a study by Ultee et al. (2000). They found a synergistic effect between combinations of carvacrol and p-cymene against the toxin production and growth of *Bacillus cereus*. Lambert et al. (2001) and Burt et al. (2005) determined an additive effect of carvacrol combined with thymol against tested bacteria. Nevertheless, Bassole and Juliani (2012) reported a synergistic effect of eugenol with carvacrol and eugenol with thymol mixture, when tested against *E. coli*.

The antimicrobial activities of the natural substances mentioned above have been well established. Therefore, we hypothesised that these compounds or their selected paired combinations would be effective against the microorganisms that contaminate boar semen. The aims of this study were to determine the antibacterial activity and toxicity for sperm cells of gallic acid, its four esters and thymol, carvacrol, eugenol and selected paired combinations of them, focusing on their application in boar semen decontamination.

MATERIAL AND METHODS

Natural substances. Thymol, carvacrol, eugenol, methyl gallate, ethyl gallate, propyl gallate and octyl gallate were purchased from Sigma Aldrich (St. Louis, MO, USA). Gallic acid was obtained from the Faculty of Pharmacy, Charles University in Hradec Kralove, Czech Republic.

Microorganisms. The bacterial strains *Enterococcus faecalis*, *Enterococcus durans*, *Staphylococcus* sp., *Escherichia coli* and *Pseudomonas aeruginosa* were isolated from native boar semen and identified on the basis of growth, Gram staining and with the use of biochemical microtest systems (Pliva Lachema, Czech Republic). The reference strains *Staphylococcus aureus* CCM 3953, *Enterococcus faecalis* CCM 4224, *Escherichia coli* CCM 3954 and *Pseudomonas aeruginosa* CCM 3955 were purchased from the Czech Collection of Microorganisms. The microorganisms were maintained on blood agar plates (Oxoid, UK) with 7% defibrinated sheep blood and cultures were stored at 4 °C and subcultured once a month when necessary.

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Culture media. Cation-adjusted Mueller-Hinton broth (Hi Media, India) was used for the susceptibility evaluation of bacterial strains tested. Blood agar plates with 5% of sheep defibrinated blood (Hi Media, India) were used for the determination of bactericidal activity of natural substances.

Antimicrobial assays. The calculated amount of natural compound was first dissolved in a small volume of 96% ethanol. After the substance was dissolved, a calculated amount of broth was added. The final concentration of ethanol in stock solution did not exceed 2% (v/v) in the experiment. The suitable ranges of natural substance concentrations used for determining susceptibility were prepared in two-fold dilutions steps.

Minimum inhibitory concentrations (MIC values) were determined using the microdilution and macrodilution method in Mueller-Hinton broth (MH broth). The bacterial inocula were prepared by emulsifying freshly subcultivated 18-hour cultures in phosphate-buffered saline (pH 7.2 ± 0.5) to the equivalent of a 0.5 McFarland turbidity scale (which corresponds to 1.5×10^8 CFU/ml) with the use of a nephelometer (Erba Lachema, Czech Republic). Inocula were subsequently diluted to 1.5×10^6 CFU/ml in sterile phosphate buffered saline. The density of the bacterial suspension after application to the wells of microtitre plates with natural substances corresponded to a yield of approximately 0.5×10^5 CFU/ml.

Macrodilution method. For the experiment, 1 ml of MH broth and 1 ml of natural substance stock solution was added to the first test-tube. After thorough stirring, 1 ml of this solution was pipeted to the second test tube containing 1 ml of MH broth. The same procedure of two-fold dilution steps was repeated up to the tenth test-tube. The eleventh test-tube containing 1 ml of MH broth and 0.05 ml of bacterial inoculum was used as a positive growth control. The twelfth test-tube served as a medium sterility control since no bacterial inoculum nor natural substance solution was added. Finally, 0.05 ml of bacterial suspension was added to each test-tube apart from the twelfth one. Inoculated test-tubes were incubated at 37 °C for 24 and 48 h, aerobically. Inhibition or growth of microorganisms was evaluated visually and three to five test-tubes showing no growth of bacteria were subcultured on a blood agar plate.

Microdilution method. The procedure for determining MIC values via the microdilution method was similar to the macrodilution method. For the experiment, 0.05 ml of Mueller-Hinton broth and 0.05 ml of natural substance stock solution were added to the first well. After thorough stirring, 0.05 ml of this solution were pippeted to the second well containing 0.05 ml of MH broth. The same procedure of two-fold dilution was repeated up to the tenth well. The eleventh well containing 0.1 ml of MH broth and 0.001 ml of bacterial inocula was used as the positive growth control. The twelfth well served as the medium sterility control since no natural substance solution and no bacterial inoculum was added. Finally, 0.001 ml of bacterial suspension were added to each well apart from the twelfth one.

Inoculated round-bottom microtitre plates were covered with a sterile lid and incubated at 37 °C for 24 and 48, aerobically. The inhibition and growth of microorganisms was evaluated visually and three to five wells of the microtitre plate showing no growth of bacteria were subcultured on a blood agar plate.

Testing combinations of natural substances. The interactions between the two natural substances were investigated using the microdilution and macrodilution method. For the microdilution method, the concentration ranges of substance A were prepared by two-fold dilutions directly in the microtitre plate. The concentration ranges of substance B were prepared separately and subsequently pippeted to the corresponding wells. The bacterial suspension (0.001 ml) was added into the wells of the microtitre plate by a microtitre inoculator. Microtitre plates were incubated for 24 and 48 h at 37 °C, aerobically. The macrodilution method for testing combinations was analogous, but with a larger volume.

The determination of the antimicrobial activity of each substance was performed in triplicate. The MIC value was defined as the lowest concentration in the wells of the microtitre plate that showed no turbidity, i.e., no visible growth of microorganisms after 24 and 48 h of incubation.

Evaluation of natural substance toxicity for sperm cells. Determination of the toxicity of natural substances for sperm cells was performed with semen collected during a two-month period from six boars. Freshly collected boar semen (1 ml) was diluted with the short-term extender (3 ml) in a ratio of 1 : 3 in test-tubes preheated to 37 °C. Subsequently, 0.1 ml of the calculated amount of natural substance dissolved in ethanol and physiologic saline was added to the test-tubes after they slowly cooled to 17 °C. After the gentle but thor-

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ough stirring of the mixture, sperm motility was observed by phase contrast microscopy. The specimen was prepared by adding 0.015 ml of the mixture to a microscope slide which was then 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 h after the addition of natural substance. Mixtures were stored for up to the 72nd hour at 17 °C.

Maximum non-toxic concentrations of natural substances were assessed on the basis of the influence on sperm motility in semen diluted in the short-term extender with various concentrations of natural substances (without antibiotics) in comparison with the control diluted semen containing no natural substances. Sperm motility was examined using phase-contrast microscopy at × 150 magnification.

RESULTS

The MIC values of natural substances determined using the macrodilution and microdilution method are given in Table 1. From the results, it is clear that thymol and carvacrol are the most effective substances at inhibiting the growth of all tested bacteria (mostly in the concentration range of 300–600 µg/ml). We found higher MIC values for eugenol (1200 to 2400 µg/ml). In the comparison of gallates, gallic acid was more effective against *P. aeruginosa* strains (MIC values of 300–1200 µg/ml). The MIC values of octyl gallate demonstrated a stronger effect on the Gram-positive than on the Gram-negative bacteria tested. Staphylococci and enterococci were inhibited by octyl gallate in the concentration range of 18.8–75 µg/ml, whereas *E. coli* and *P. aeruginosa* were inhibited in the ranges of 300–600 µg/ml and 1200–2400 µg/ml, respectively.

The MIC values of gallic acid combined with carvacrol, thymol, eugenol and octyl gallate are listed in Table 2, ethyl gallate and methyl gallate with thymol and carvacrol in Table 3. The remaining mutual combinations of thymol with carvacrol or eugenol and carvacrol with eugenol are presented in Table 4.

From the results listed in Table 4, it is evident that thymol combined with carvacrol was the most effective mixture of natural substances against both the Gram-positive and Gram-negative bacteria in this study. The MIC values of the thymol and carvacrol combinations determined using the macrodilution method were 300 : 300 µg/ml (600 : 600 µg/ml). The MIC values obtained using the microdilution method were slightly lower than with the macrodilution

Table 1. Ranges of minimum inhibitory concentrations (µg/ml) of natural substances determined using the macrodilution (D) and microdilution (M) methods against tested bacterial strains

Natural substances		*Pseudomonas aeruginosa* (*n* = 5)	*Escherichia coli* (n = 3)	*Staphylococcus* sp. (*n* = 4)	*Enterococcus* sp. (*n* = 5)
Gallic acid	D	600–1200 (2400)	2400–4800	2400–4800	2400–4800
	M	300–600	4800	≥ 4800	2400–4800
Methyl gallate	D	600–1200 (2400)	1200–2400	1200–2400 (4800)	2400–4800
	M	N	N	N	N
Ethyl gallate	D	600–1200 (2400)	600–1200 (2400)	600–2400	2400–4800
	M	N	N	N	N
Propyl gallate	D	1200–2400	600–2400	1200–2400 (4800)	1200–4800
	M	N	N	N	N
Octyl gallate	D	N	N	N	N
	M	1200–2400	300–600	18.8–37.5	37.5–75
Carvacrol	D	300–600 (1200)	150–300 (600)	(150) 300–600	300–600 (1200)
	M	(150) 300–1200 (2400)	75–300	300–600 (1200)	300–600
Thymol	D	300–600 (1200)	150–300	300–600	300–600
	M	(150) 600–1200 (2400)	300(600)	300–600	300–600 (1200)
Eugenol	D	1200–2400 (4800)	(300) 600–1200	1200 (2400)	1200–2400
	M	1200–2400 (4800)	600–1200	2400	600–2400

Values occasionally found in repeated determination of MIC values are in parentheses
N = not performed

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Table 2. Ranges of minimum inhibitory concentrations (μg/ml) of gallic acid combined with carvacrol, thymol, eugenol and octyl gallate determined using the macrodilution (D) and microdilution (M) methods against tested bacterial strains

Combinations		*Pseudomonas aeruginosa* (*n* = 5)	*Escherichia coli* (*n* = 3)	*Staphylococcus* sp. (*n* = 4)	*Enterococcus* sp. (*n* = 5)
Gallic acid : carvacrol	D	1200 : 1200	1200 : 600	1200 : 600	2400 : 1200
	M	37.5–75 : 600 300 : 300	N	N	75 : 600 150 : 300 1200 : 300–600
Gallic acid : thymol	D	1200 : 1200	1200 : 600	1200 : 600	600 : 600 1200 : 600–1200
	M	75 : 600 150 : 300	N	N	75 : 300 1200 : 150–300 2400 : 37.5–150
Gallic acid : eugenol	D	1200 : 1200	1200 : 1200	1200 : 1200	1200 : 1200
	M	75 : 1200 300 : 600	N	N	1200 : 1200 2400 : 600
Gallic acid : octyl gallate	D	N	N	N	N
	M	150 : 2400 300 : 300–600 600 : 300	N	N	150–2400 : 300

N = not performed

method, i.e. 75–300 : 300 μg/ml and 300 : 75–300 μg/ml for *P. aeruginosa* strains, 37.5–75 : 300 μg/ml and 150 : 37.5–150 μg/ml for enterococci.

From the results of the sperm toxicity evaluation presented in Table 5, it is clear that the natural substances thymol, carvacrol and eugenol were the least

Table 3. Ranges of minimum inhibitory concentrations (μg/ml) of gallates combined with thymol and carvacrol determined using the macrodilution (D) and microdilution (M) methods against tested bacterial strains

Combinations		*Pseudomonas aeruginosa* (*n* = 5)	*Escherichia coli* (*n* = 3)	*Staphylococcus* sp. (*n* = 4)	*Enterococcus* sp. (*n* = 5)
Ethyl gallate : thymol	D	300 : 300–600 600 : 600	150 : 150–300 300 : 150–300	150 : 150 300 : 300	300 : 300 600 : 600
	M	300 : 300–600 600 : 300–600	N	N	150–2400 : 300
Ethyl gallate : carvacrol	D	300 : 300 600 : 600	150 : 150 300 : 300	150 : 150 300 : 300	300 : 300
	M	150–300 : 1200 300 : 150 600 : 300 1200 : 37.5	600 : 37.5–150 1200 : 75	600 : 150 2400 : 37.5	1200 : 150 2400 : 37.5
Methyl gallate : thymol	D	300 : 300 600 : 600	150 : 150 300 : 300	150 : 150 300 : 300	300 : 300 600 : 600
	M	N	N	N	N
Methyl gallate : carvarol	D	600 : 600	300 : 300	300 : 300	300 : 300 600 : 600
	M	75 : 300–1200 150 : 300–600 300 : 150–300 600 : 37.5	150 : 150–300 300 : 37.5–300 600 : 75–150	150 : 75 1200 : 37.5	37.5 : 600 1200 : 150

N = not performed

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Table 4. Ranges of minimum inhibitory concentrations (μg/ml) of natural substance combinations determined using macrodilution (D) and microdilution (M) methods against tested bacterial strains

Combinations		*Pseudomonas aeruginosa* (*n* = 5)	*Escherichia coli* (*n* = 3)	*Staphylococcus* sp. (*n* = 4)	*Enterococcus* sp. (*n* = 5)
Thymol : carvacrol	D	300 : 300 600 : 600	300 : 300	300 : 300	300 : 300 600 : 600
	M	75–300 : 300 300 : 75–150	37.5–150 75 : 75 150 : 37.5	37.5 : 300 75 : 75 150 : 37.5	37.5–75 : 300 75–300 : 150 150 : 37.5–150 300 : 37.5–300
Thymol : eugenol	D	300 : 300 600 : 600	300 : 300 600 : 600	300 : 300 600 : 600	300 : 300 600 : 600
	M	150–300 : 1200 600 : 300–1200 1200 : 37.5–150	75 : 150–300 150 : 75	150 : 150–300 300 : 75	75–150 : 600 300 : 300 600 : 75
Carvacrol : eugenol	D	600 : 600 (1200 : 1200)	300 : 300	300 : 300	300 : 300 600 : 600
	M	75–150 : 1200 300 : 600–1200 600 : 300 1200 : 75–300	37.5 : 150 75–150 : 75 150 : 37.5–150	75–150 : 300 75–150 : 600 300 : 150–300	37.5–300 : 300 300 : 150–300

toxic of the tested compounds for sperm cells at a concentration of 300 μg/ml. 10–20% motile sperms were observed in boar semen diluted by the semen extender without antibiotics at a concentration of 300 μg/ml of these natural substances after 72 h.

DISCUSSION

Experience with the use of extended semen for the artificial insemination of farm animals hase shown that the biological quality of insemination doses may be negatively affected by microorganisms contaminating the ejaculate during semen collection and subsequent processing. The composition of seminal plasma and storage conditions enable the successful multiplication of microorganisms. The harmful effects of microbes on sperm cells are facilitated in particular through the products of metabolism, exotoxins, endotoxins and destruction of energy sources located in seminal plasma by bacteria, which leads to a decrease in the survivability and fertilising ability of spermatozoa. These findings justify the necessity of eliminating microorganisms which contaminate ejaculates. Various antibiotics or combinations of them are still used to suppress the growth of these microorganisms. As mentioned above, the long-term use of antimicrobial agents causes an increasing resistance of microorganisms, which is a serious problem for the treatment of infectious diseases in developing and also developed countries. Resistant bacteria are not only found in hospitals, where a large amount of antibiotics are used, but also in farms and farm animal products. Occurrence of multi-resistant *Salmonella* Typhimurium strains isolated from pigs may serve as a good example (Sisak et al. 2006). As a result, veterinary workers are expending considerable effort towards restricting or even prohibiting the use of antibiotics for non-therapeutic purposes – (Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22nd September 2003 on additives for use in animal feeds). Due to the above, our study was focused on investigating the antibacterial activity of natural compounds with the aim of their possible utilisation for decontaminating boar insemination doses.

We found gallic acid to be the most effective substance against *P. aeruginosa*, with MIC values in the range of 300–1200 μg/ml (see Table 1). Binutu and Cordell (2000) determined an MIC value of 1000 μg/ml for gallic acid against *P. aeruginosa*. According to the studies by Ozcelik et al. (2011) and Al-Zahrani (2012) who reported lower MIC values, gallic acid is an effective substance against this bacterium, and that is in accordance with our data. The ranges of MIC values against other bacteria tested were 2400–4800 μg/ml (refer to Table 1).

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Table 5. Evaluation of the toxicity of natural substances for sperm determined at various time intervals

Natural substances	Concentration (µg/ml)	Motile sperm cells[a] (%)					
		0	1	4	24	48	72
		hour					
Gallic acid	300	75	65	75	30	0	0
	600	70	60	70	25	0	0
	1200	60	70	60	25	0	0
	2400	50	65	60	40	10	0
Methyl gallate	300	50	70	65	50	40	30
	600	20	45	55	35	20	15
	1200	< 5	< 5	20	0	0	0
Ethyl gallate	300	30	50	60	50	40	40
	600	0	5	30	5	5	5
	1200	0	0	0	0	0	0
Propyl gallate	300	0	< 5	5	10	10	10
	600	0	0	0	0	0	0
	1200	0	0	0	0	0	0
Octyl gallate	150	60	0	0	0	0	0
	300	60	30	20	0	0	0
Carvacrol	150	55	60	65	40	40	40
	300	60	40	50	40	20	15
	600	0	0	0	0	0	0
Thymol	150	75	60	60	25	20	10
	300	50	55	50	15	15	10
	600	0	0	0	0	0	0
Eugenol	300	70	55	60	40	30	20
	600	70	55	20	15	0	0
	1200	50	30	0	0	0	0
	2400	30	5	0	0	0	0
1% ethanol[b] (v/v)		80	80	80	60	40	35
Control[c]		80	80	80	65	40	40

[a]motility values are expressed as median of six different boar semen samples at each time interval

[b]boar semen diluted by BSA extender with ethanol added to a final concentration of 1% (v/v) without antibiotics

[c] boar semen diluted by BSA extender without antibiotics

The antibacterial activity of gallic acid against methicillin-resistant *S. aureus* strains was determined in a study by Chusri and Voravuthikunchai (2011) who reported minimum inhibitory concentration (MIC) values against this species of 60 µg/ml. Al-Zahrani (2012) reported MIC values in the range of 3.5–12.5 µg/ml against methicillin-resistant *S. aureus*. Kang et al. (2008) investigated the susceptibility of *Streptococcus mutans*, *Lactobacillus casei*, and *Lactobacillus acidophilus* to gallic acid and found an MIC value of 8000 µg/ml using the microdilution method. Furthermore, Nohynek et al. (2006) reported good antibacterial activities of gallic acid against *Helicobacter pylori*, *Bacillus cereus*, and *Salmonella* sp. Gallic acid is highly soluble in water; however, one disadvantage is that gallic acid solutions gradually darken from colourless to brown. Octyl gallate was the most potent substance against Gram-positive bacteria among the alkyl gallates tested, exhibiting MIC values in the range of 18.8–75 µg/ml. The results are presented in Table 1.

Thymol and carvacrol exhibited MIC values of 150–1200 µg/ml for *E. coli*, *Staphylococcus* sp., and *Enterococcus* sp. strains as determined using

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the macrodilution and microdilution methods (see Table 1). These results are in agreement with those previously reported by the majority of authors cited. Lower MIC values of 50–250 µg/ml were reported by Didry et al. (1993) and Giweli et al. (2012) for both substances. For *S. aureus*, including methicillin-resistant strains, MIC values of thymol in the concentration range of 100–580 µg/ml were reported (Janseen et al. 1986; Cosentino et al. 1999; Trombetta et al. 2005; Cristani et al. 2007; Zarrini et al. 2010; Guarda et al. 2011; Rua et al. 2011; Solorzano-Santos and Miranda-Novales 2012; Wattanasatcha et al. 2012). MIC values of carvacrol for *S. aureus* strains in the ranges of 125–450 µg/ml (Solorzano-Santos and Miranda-Novales 2012), 250–1000 µg/ml (Janseen et al. 1986), 1250 µg/ml (Cristani et al. 2007) and 1700–1800 µg/ml (Veldhuizen et al. 2006) have been reported.

In contrast, Tippayatum and Chonhenchob (2007) determined significantly higher MIC values of 3000–5000 µg/ml for *S. aureus* and *E. coli* using the agar dilution method. In our study, thymol and carvacrol also exhibited inhibitory activity against *P. aeruginosa* strains, with MIC values ranging from 300–2400 µg/ml (see Table 1). According to results published in studies by Janseen et al. (1986) and Walsh et al. (2003), MIC values for thymol and carvacrol ranged from 2000–8000 µg/ml for *P. aeruginosa*. MIC values of thymol reported in other studies were more than 500 µg/ml for *P. aeruginosa* strains (Cosentino et al. 1999; Walsh et al. 2003). In contrast, Lambert et al. (2001), Zarrini et al. (2010) and Wattanasatcha et al. (2012) demonstrated MIC values of thymol in the range of 380–400 µg/ml for *P. aeruginosa* strains.

Eugenol is considered to be a natural substance with a broad-spectrum efficacy against Gram-positive and Gram-negative bacteria (Dorman and Deans 2000). We determined MIC values of 1200–2400 µg/ml for *P. aeruginosa*, *E. faecalis*, and *E. durans* strains (see Table 1). Similar results for *P. aeruginosa* were reported by Medina et al. (2009) and Joshi et al. (2013). A significantly lower MIC value of 273 µg/ml was reported by Bassole et al. (2010). Tippayatum and Chonhenchob (2007) reported an MIC value of 800 µg/ml for *S. aureus* and Hammer and Heel (2012) found a value of 200 µg/ml for *Enterococcus faecalis*. Antibacterial properties against Gram-positive and Gram-negative bacteria have also been reported in a study by Palaniappan and Holley (2010), who determined MIC values of 410 µg/ml for both *S. aureus and E. coli* strains whereas Walsh et al. (2003) reported an MIC value of 1067 µg/ml for *S. aureus* and 530 µg/ml for *E. coli*.

The testing of paired combinations of natural substances was aimed at evaluating their mutual actions such as an enhancement or extension of antimicrobial activity. This could enable the minimum inhibitory concentrations to be decreased with no loss of antibacterial efficacy. The MIC values of the combinations of natural substances determined using the microdilution method differed slightly from those determined using the macrodilution method, especially for combinations with gallic acid. This is probably due to discrepancies in the methodology and solubility of natural substances in small and large volumes.

We did not find any significant synergistic effects between combinations of gallic acid with thymol, carvacrol, eugenol and octyl gallate. Most of the MIC values for the natural substances tested as single compounds correlated with those determined in combinations (refer to Table 1 and Table 2). Similar results were shown for combinations of gallates with thymol and carvacrol.

The most effective combination was thymol with carvacrol, with MIC values of 75–300 : 300 and 300 : 75–150 µg/ml (thymol : carvacrol) for *P. aeruginosa* strains and 75–300 : 150 and 150 : 37.5–150 µg/ml (thymol : carvacrol) for the enterococcal strains included in our study (see Table 4). Both of these microorganisms are well known as pathogens with a natural resistance to many antibiotics. Furthermore, these bacterial strains often acquire resistance against different classes of antibiotics via horizontal gene transfer or spontaneous mutations. Although some of the MIC values of thymol and carvacrol tested in combination were lower than the MIC values of single substances, these mixtures were found to produce an additive rather than a synergistic effect against the bacterial strains tested. Both carvacrol and thymol have a similar chemical structure, differing only in the location of a hydroxyl group on the benzene ring and they share an analogous mechanism of action against microorganisms, which may explain their almost identical MIC values and lack of any synergistic effects. Lambert et al. (2001) and Burt et al. (2005) have reported similar results for a combination of thymol with carvacrol.

The MIC values of carvacrol and eugenol combinations were 37.5–300 : 150–300 µg/ml for the enterococci and staphylococci tested (see Table 4).

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In our study, we did not find any synergistic effect of this mixture for the above microorganisms. However, Bassole and Juliani (2012) found that eugenol combined with carvacrol or thymol had synergistic effects against the bacterium *E. coli*. These authors reported that thymol and carvacrol damage the bacterial membrane and enable the penetration of eugenol into the bacteria cell.

The utilisation of natural substances for boar semen decontamination may be limited by the toxicity of natural substances for sperm cells. Natural substances as well as antibiotics may negatively affect the motility and the viability of sperm cells. Hence, we examined combinations of natural substances. However, most of the results in this study only indicate an additive effect of the combinations tested. Nevertheless, we found that the biological quality of sperm cells was not significantly influenced by the concentrations of thymol and carvacrol applied individually, which were effective against the bacterial strains tested. These results suggest the potential application of carvacrol and thymol for boar semen decontamination.

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Corresponding Author:

Rudolf Kukla, University of Pardubice, Faculty of Chemical Technology, Pardubice, Czech Republic
E-mail: rudolf.kukla@tiscali.cz