

## ORIGINAL ARTICLE

# ***IN VITRO* ANTIMICROBIAL ACTIVITY OF NATURAL SUBSTANCES CONVENIENT FOR USE IN ANIMAL BREEDING INSTEAD OF ANTIBIOTICS**

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Received 14<sup>th</sup> June 2019.

Accepted 31<sup>st</sup> July 2019.

Published 6<sup>th</sup> March 2020.

### Summary

The increasing antibiotic resistance of microbial pathogens isolated from farm animals tissues and the environment has been the one of the most important challenges associated with the use of antibiotics. In order to achieve better production on a farm, animal feed is enriched with antibiotics often originally intended for therapeutic purposes, which may lead to notable increases in microbial resistance. One possible approach to decreasing the excessive use of antibiotics in livestock as well as antimicrobial resistance is utilizing the antimicrobial properties of natural substances.

The aim of this study was to evaluate the antimicrobial activity of natural substances including carvacrol, thymol, eugenol, gallic acid, octyl gallate, cnicin and usnic acid against a wide spectrum of microorganisms. Cnicin was the only compound which was isolated from the plant with use of column chromatography. The antimicrobial activities of these natural substances were determined on the basis of their minimum inhibitory, minimum bactericidal and minimum fungicidal concentrations using the microdilution method.

This determination of antimicrobial activity revealed thymol and cnicin to be effective natural substances against all tested microorganisms. Octyl gallate had a strong inhibitory and bactericidal effect against gram-positive bacteria and was the most effective against *Candida* strains. Usnic acid was shown to have the lowest minimum inhibitory concentrations for gram-positive bacteria. These results suggest the possible incorporation of natural substances in animal rearing in order to reduce the high amount of antibiotics which are not used directly to treat animal diseases.

**Key words:** *Natural substances; antimicrobial activity; bacteria; candida*

## INTRODUCTION

The intensive therapeutic use of antibiotics for humans and animals has been responsible for a strong selective pressure facilitating the rise and spread of the bacteria with antimicrobial resistance (Lauterwein et al., 1995; Segatore et al., 2012). It can be clearly illustrated with the evolution of *Staphylococcus aureus* (*S. aureus*) resistance to penicillin, where the first report about the production of penicillinase was mentioned in 1944 by Kirby (1944). Resistance against methicillin, the first semisynthetic penicillinase-resistant beta-lactam antibiotic, developed rapidly after its introduction in 1961 (Lowy, 2003).

The increasing antibiotic resistance of microorganisms isolated from farm animals tissues and the environment has been one of the most important global challenges associated with the use of antibiotics. In order to facilitate satisfactory farm animal production, an animal feed is supplemented with antimicrobial agents at subtherapeutic concentrations to enhance growth, increase feed efficiency and to prevent infection (Wegener et al., 1999; Palaniappan and Holley, 2010). As reported by Van Den Bogaard and Stobberingh (2000), approximately 30% of all antibiotics intended for farm animals had been used as antimicrobial growth promoters or performance enhancers in Europe. However, the use of antimicrobial agents as growth promoters ended in the European Union on January 1, 2006 (Gaggia et al., 2007; Opletal et al., 2007; Kilic et al., 2011). In the United States, 50% of all antibiotics produced are administered to farm animals, especially for subtherapeutic purposes (Palaniappan and Holley, 2010). The high selective pressure due to the use of antimicrobial agents as growth promoters may contribute to the presence and rise of resistant microorganisms in farm animals (Wegener et al., 1999). The animal-to-human transmission of resistant microbes is possible via the food chain or farmers' contact with farm animals and animal waste (Palaniappan and Holley, 2010). The assumption that the use of antibiotics as feed additives generated resistant bacteria was confirmed in enterococci due to the use of the glycopeptide avoparcin (Birkegård et al., 2019). Moreover, a plasmid-mediated colistin resistance encoded by *mcr* genes has been reported frequently from animal production in China. Zhang et al. 2019 reported 27 – 54% percentage of colistin resistant *Escherichia coli* strains from rectal swabs of pigs, chickens and cattle. A prevalence of *mcr* genes was almost twice as high as the percentage of colistin resistant *Escherichia coli* (Zhang et al. 2019). As a result, the Food and Drug Administration released a proposal guidance recommending a restriction on the use of antibiotics in animal agriculture (Looft et al., 2012).

One possible alternative that could enhance the defence mechanisms of farm animals and simultaneously reduce the excessive use of antibiotics connected to the increase in microbial resistance, is the utilization of natural substances such as terpenes (germacranolides), simple phenolic compounds and many others secondary metabolites of plants or lichens. Natural substances are attracting attention for their antibacterial, antiviral, antifungal, antiparasitical, antioxidant and other properties (Bakkali et al., 2008; Kilic et al., 2011; Rozkot et al., 2013).

In a previous study, we evaluated the antimicrobial effects of more than 40 natural substances and natural extracts from natural sources. Out of these substances, we selected the seven most effective including thymol, carvacrol, eugenol, gallic acid, octyl gallate, knicin and usnic acid on the basis of our results and available data from other studies. The antimicrobial activities of these seven natural substances were investigated against a broad spectrum of aerobic bacteria, anaerobic bacteria and also against *Candida* strains.

Thymol and carvacrol are phenolic isomeric monoterpenes that differ in the location of the hydroxyl group on the ring. Thymol (2-isopropyl-5-methylphenol) is regioisomer isomer of carvacrol (5-isopropyl-2-methylphenol). These compounds are common constituents of essential oils, especially those derived from *Thymus* and *Origanum* plants (Evans and Martin, 2000; Nostro et al., 2004; Guo et al., 2009; Brewer, 2011). The antimicrobial activity of these substances against both gram-positive and gram-negative bacteria has been demonstrated in many previous studies (Nostro et al., 2004). Antifungal and anticandidal activity have been also reported (Guo et al., 2009). The mechanism of their antimicrobial action is mainly based on their ability to disturb membrane permeability and membrane potential (Gill and Holley, 2004; Xu et al., 2008; García-García et al., 2011).

Eugenol is also a naturally occurring aromatic phenolic substance found in a significant concentration in the clove bud oil from *Syzygium aromaticum* (L.) MERR. & L. M. PERRY and is known to be a flavoring agent in food and cosmetics (Devi et al., 2010; Qiu et al., 2010). Many publications have reported an antibacterial activity of eugenol against bacteria including *Escherichia coli* (*E. coli*), *S. aureus*, *Pseudomonas aeruginosa* (*P. aeruginosa*),

*Listeria monocytogenes* and *Salmonella typhi* (Gill and Holley, 2004; Oussalah et al., 2007; Pei et al., 2009; Devi et al., 2010). In addition, eugenol has pronounced antioxidant activity, especially in terms of the inhibition of lipid peroxidation and also anti-inflammatory activity (Ogata et al., 2000; Qiu et al., 2010).

Gallic acid, also known as trihydroxybenzoic acid, is a simple phenolic acid that is an important part of hydrolysable tannins, found widely distributed in many fruits and plants, characterized by higher solubility in water compared to the other tested compounds (Ow and Stupans, 2003; Soobrattee et al., 2005). Gallic acid also possesses antimicrobial activity against various species of bacteria and fungi (Kang et al., 2008; Cueva et al., 2012; Nguyen et al., 2013). Furthermore, gallic acid acts as a strong antioxidant (Soobrattee et al., 2005). Its antibacterial properties are most likely associated with its bacterial membrane disintegrating activity (Nohynek et al., 2006).

Octyl gallate is an ester of the ubiquitously occurring natural substance gallic acid. This compound is primarily known for its pronounced antioxidant properties (Ha et al., 2004). Nevertheless, octyl gallate possesses significant antifungal properties and also strong antibacterial effects against gram-positive bacteria and some gram-negative bacteria (Kubo et al., 2001). An antiviral effect against DNA as well as RNA viruses was also determined (Uozaki et al., 2007). Antibacterial and antifungal activities are associated with a balance between the hydrophobicity of the side chain and the hydrophilicity of its hydroxyl groups on the benzene ring. Octyl gallate acts as nonionic surfactant and is able to inhibit efflux pumps (Kubo et al., 2004; Kubo et al., 2010; Rangel et al., 2010; Chew et al., 2019).

Cnicin is a natural substance belonging to the abundant group of sesquiterpen lactones found in the plant *Cnicus benedictus* L (Karioti et al., 2002; Bachelier et al., 2006; Bugg et al., 2011). Cnicin has significant antibacterial and antifungal effects. Interestingly cnicin, as a substance derived from plants, exhibits a unique mechanism of action because it is able to inhibit peptidoglycane biosynthesis (Steinbach et al., 2008; Bugg et al., 2011).

Usnic acid is a widespread dibenzofuran derivative found as a secondary metabolite in lichens. Usnic acid has been studied since the 1950s, mainly in terms of its antimicrobial effect. Usnic acid possesses strong antimicrobial activity against a wide variety of gram-positive bacterial pathogens including *S. aureus*, *Enterococcus faecalis*, beta-hemolytic streptococci and clostridia (Cocchietto et al., 2002; Francolini et al., 2004; Sundset et al., 2008). Many studies have investigated other properties of this compound, including its antimycobacterial, antiviral, antioxidative, antiproliferative and anti-inflammatory activities (Cocchietto et al., 2002).

## MATERIALS AND METHODS

### Microbial strains

Microbial strains used as test organisms were either obtained from the Czech Collection of Microorganisms: *Bacillus subtilis* CCM 2215, *Enterococcus faecalis* CCM 4224, *Listeria monocytogenes* CCM 5576, *Staphylococcus aureus* CCM 4223, *Alcaligenes faecalis* CCM 1052, *Escherichia coli* CCM 3954, *Bacteroides fragilis* CCM 4508 and *Candida albicans* CCM 8186, isolated from boar semen: *Providencia stuartii*, *Pseudomonas aeruginosa*, *Streptococcus porcinus* and *Candida catenulata* or obtained from the University of Pardubice collection: *Klebsiella pneumoniae* and *Clostridium perfringens*.

Bacterial strains were maintained on blood agar plates (Hi Media, India) and cultures were stored at 4°C and subcultured once a month when necessary. *Candida* strains were maintained on malt agar plates (Hi Media, India), stored at 4°C and subcultured once a month when necessary.

### Culture media

Cation-adjusted Mueller-Hinton broth (Hi Media, India) was used for the susceptibility evaluations of most of the bacterial strains tested. Brain Heart Infusion broth (BHI) from (Hi Media, India) was used for *Streptococcus porcinus*. Sabourad-dextrose broth (Hi Media, India) was used for the susceptibility testing of *Candida* strains. Blood agar plates (Hi Media, India) were used for determining the bactericidal activity of natural substances. Sabourad-dextrose agar plates were used for determining the fungicidal activity of natural substances.

## Natural substances and antimicrobials

The natural substances apart from cnicin were purchased as pure components. Thymol, carvacrol, eugenol, gallic acid and octyl gallate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Usnic acid was obtained from Carl-Roth (Germany). Cnicin was obtained via the extraction and isolation process mentioned below. The antimicrobial agent ampicillin was obtained from Biotika (Slovakia), amfotericin B from Sigma-Aldrich (St. Louis, MO, USA) and gentamicin from Dr. Kulich Pharma (Czech Republic).

## Extraction and isolation of cnicin

### Plant material

Blessed thistle herb (*Cnicus benedictus* L.) was obtained from Nativia (Czech Republic). A voucher specimen (No. 15-274-01) identified by L. Opletal was deposited in the Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy, Charles University, Hradec Kralove.

### Chemicals and procedures

Solvents and chemicals (ethanol EtOH, methanol MeOH, chloroform  $\text{CHCl}_3$ , dichloromethane  $\text{CH}_2\text{Cl}_2$ , petroleum ether, petrol, sulphuric acid 95% and vanillin pure) were obtained from PENTA (Czech Republic). The chromatographic adsorbents 100-200  $\mu\text{m}$  Silica gel, deactivated with 10% water for column chromatography and UV 254 nm Silica gel TLC plates were purchased from Sigma-Aldrich (St. Louis, MO, USA). All extraction procedures were performed under reduced pressure and at a temperature of 40 °C. The substance was always dried in a vacuum desiccator (1.33 kPa).

### Extraction and isolation procedure

3.34 kg of pulverized blessed thistle herb was percolated with 70% EtOH (45 litres of extract). The alcohol was distilled under reduced pressure at 40 °C, the concentrated water extract (4 L) was filtered and exhaustively extracted with  $\text{CHCl}_3$  (6x750 mL). The organic phase was evaporated (32 g of deep green very viscous residue), and this crude extract was dissolved in 600 mL of 80% MeOH (v/v) and purified by petroleum ether extraction (7x120 mL). The deep green MeOH solution was evaporated (24.6 g).

This residue was further fractionated in a silica gel column (6x73 cm,  $\text{CHCl}_3$ +petrol 80:20,  $\text{CHCl}_3$ ,  $\text{CHCl}_3$ +increasing concentration of EtOH, 250 mL, 97 fractions) under TLC control (Silica gel plates UV 254 nm, line 10 cm,  $\text{CH}_2\text{Cl}_2$ +MeOH 90:10) developed twice and detected with a vanillin-sulphuric acid reagent, 80 °C, (cnicin:  $R_f$  0.28, blue). Fractions 47-59 were combined and the solvent evaporated ( $\text{CHCl}_3$ +EtOH 95:5) to obtain 3.28 g of white fine needle-like substance. This was recrystallized from aqueous EtOH to obtain 2.16 g of white, needle-like substance.

### Identification of cnicin

Melting point 142-143 °C (Kofler), optical purity was determined by specific rotation  $[\alpha]_D^{21} = +146$  (c=2.30, EtOH). Elemental analysis: found C 65.58%; H 7.08%; calculated C 65.91%, H 7.19%.

Structural elucidation ( $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra) and other physical data were compared with a reference substance of cnicin ROTICHROM (No. 4560.1) obtained from Carl Roth (Germany); no significant differences were found.

## Antimicrobial assay

For the experiment, all natural compounds were first dissolved in a small amount of 96% ethanol (EtOH). After the dissolution of the substance, a calculated amount of broth was added. The final concentration of EtOH in the stock solution did not exceed 1.0% (v/v) in the experiment. Stock solutions of antimicrobials (gentamicin,

ampicillin, amphotericin B) were prepared by dissolving in sterile redistilled water. After the dissolution of the antimicrobial, a calculated amount of broth was added. The suitable concentration ranges of the natural substances and antimicrobials used to determine susceptibility were prepared in two-fold dilution steps. The concentration ranges of the natural substances, antimicrobials and EtOH are given in TABLE 1.

**TABLE 1.** Concentration ranges of natural substances, antimicrobial agents and ethanol used for determination of antimicrobial activity

Tested compound	Concentration range (mg.L <sup>-1</sup> )	For
Carvacrol	37.5 - 4800	All microorganisms
Thymol	37.5 - 4800	All microorganisms
Eugenol	37.5 - 4800	All microorganisms
Gallic acid	37.5 - 4800	All microorganisms
Octyl gallate	4.7 - 600 37.5 - 4800	Gram-positive bacteria and <i>Candida</i> strains Gram-negative bacteria
Cnicin	18.8 - 2400	All microorganisms
Usnic acid	1.2 - 600 75 - 9600	Gram-positive bacteria Gram-negative bacteria, <i>Candida</i> strains
Gentamicin	0.5 - 64 0.015 - 4	<i>Streptococcus porcinus</i> , <i>Enterococcus faecalis</i> , <i>Providencia stuartii</i> Other bacteria than above mentioned
Ampicillin	0.03125 - 16 1 - 512	Gram-positive bacteria Gram-negative bacteria
Amphotericin B	0.03 - 4	<i>Candida</i> strains
Ethanol <sup>a</sup>	0.375 - 48 %	All microorganisms

<sup>a</sup> concentration of ethanol is in % (v/v)

Minimum inhibitory concentrations (MICs) were determined by the microdilution method in Mueller-Hinton broth. BHI broth was used for *Streptococcus porcinus*, Sabourad-dextrose broth for *Candida* strains. The bacterial inocula and the inocula of *Candida* strains were prepared by emulsifying freshly subcultivated 24 hours cultures in physiological saline to the equivalent of a 0.5 McFarland turbidity scale (responses 1.5x10<sup>8</sup> CFU/ml) using a nephelometer (Erba Lachema, CZ). Microbial inocula were subsequently diluted in sterile physiological saline. The density of the bacterial and candidal suspension after application to wells of microtiter plates with natural substances corresponded to a yield of approximately 0.5x10<sup>5</sup> CFU.mL<sup>-1</sup> and 2.5 x 10<sup>3</sup> CFU.mL<sup>-1</sup>, respectively.

After density adjustment, the microbial suspension was used within 15 min, because the number of viable microorganisms might otherwise change. Inoculated round-bottom microtiter plates were covered with sterile lid and incubated at 37 °C for 24, 48 and 72 hours, aerobically. Anaerobic bacteria were incubated in AnaeroGen Compact (Oxoid, UK) at 37 °C for 24, 48 and 72 hours.

The MIC value was defined as the first well that showed no visible growth of microorganisms after 24, 48 and 72 hours of incubation. The minimum bactericidal concentration (MBC value) was determined by subsequent subcultivations of 3 to 5 wells of microtiter plate exhibiting no growth of the microorganisms. The contents of the corresponding wells were inoculated with a 1 µl calibrated bacterial loop on sterile blood agar plates for bacteria and on sterile Sabourad dextrose agar plates for *Candida* strains. The MBC value was defined as the lowest concentration of the natural substance, i.e., the first concentration without any observed growth of colonies, e.g. at least 99.9% of bacteria was killed.

The determination of the antimicrobial activity of each substance, including antimicrobials and EtOH, was performed in triplicate. The MIC, MBC and MFC values were presented as median values. Growth controls and sterility of the medium controls were performed simultaneously with the determination of antimicrobial activity. The growth control was a broth containing 1.0% of EtOH (v/v) where the corresponding microbial suspension was pipetted. The sterility of the medium control was a sterile broth.



## RESULTS AND DISCUSSION

In our work, thymol and carvacrol exhibited inhibitory activity against all the tested microorganisms. In addition, the differences among the MICs and MBCs were no more than 2-fold for individual microorganisms, suggesting that the activity of thymol and carvacrol is both bactericidal and fungicidal (TABLES 2,3,4). We found thymol to be more effective against *K. pneumoniae* with an MIC of 150 mg.L<sup>-1</sup> and MBC of 300 mg.L<sup>-1</sup> than carvacrol after 24 hours of incubation, whereas carvacrol was more effective against *E. coli* (MIC 75 mg.L<sup>-1</sup>; MBC 75 mg.L<sup>-1</sup>), *P. aeruginosa* (MIC 150 mg.L<sup>-1</sup>; MBC 300 mg.L<sup>-1</sup>) and *Candida* strains (MIC 75-150 mg.L<sup>-1</sup>; MBC 150 mg.L<sup>-1</sup>). These results indicate that thymol and carvacrol possess a broad antimicrobial spectrum. Nostro et al. (2004) reported the MIC values of thymol and carvacrol against *S. aureus* 600 mg.L<sup>-1</sup> and 150-300 mg.L<sup>-1</sup>, respectively, using an agar dilution method. These results are similar to the results of our work (see TABLE 2). Zarrini et al. (2010) found that the MIC values of thymol against *S. aureus*, *Bacillus cereus* and *P. aeruginosa* were 200, 100 and 400 mg.L<sup>-1</sup>, respectively, using a microdilution method. This is in accordance with our results. Palaniappan and Holley (2010) found thymol to have a weaker activity against *S. aureus* and *Streptococcus pyogenes* than carvacrol.

**TABLE 2.** Minimum inhibitory concentrations and minimum bactericidal concentrations (mg.L<sup>-1</sup>) of natural substances determined for tested gram-positive bacteria after 24 and 48 hours

Microorganisms		Tested compounds						
		Carvacrol	Thymol	Eugenol	Gallic acid	Octyl gallate	Cnicin	Usnic acid
<i>Bacillus subtilis</i>	<sup>24</sup> MIC(MBC) <sup>a</sup>	300(300)	150(150)	2400(2400)	4800(4800)	18.8(18.8)	150(600)	4.7(9.4)
	<sup>48</sup> MIC(MBC) <sup>b</sup>	300(300)	150(150)	2400(2400)	4800(4800)	18.8(18.8)	600(600)	4.7(9.4)
<i>Enterococcus faecalis</i>	<sup>24</sup> MIC(MBC)	300(600)	300(600)	2400(2400)	4800(<4800)	75(75)	75(600)	18.8(N <sup>c</sup> )
	<sup>48</sup> MIC(MBC)	600(600)	600(600)	2400(2400)	4800(<4800)	75(75)	600(600)	18.8(N)
<i>Staphylococcus aureus</i>	<sup>24</sup> MIC(MBC)	300(600)	300(600)	2400(2400)	4800(<4800)	18.8(37.5)	300(600)	9.4(N)
	<sup>48</sup> MIC(MBC)	600(600)	300(600)	2400(2400)	4800(<4800)	18.8(37.5)	300(600)	9.4(N)
<i>Streptococcus porcinus</i>	<sup>24</sup> MIC(MBC)	300(300)	600(600)	1200(1200)	4800(4800)	18.8(18.8)	300(300)	4.7(N)
	<sup>48</sup> MIC(MBC)	300(300)	600(600)	1200(1200)	4800(4800)	18.8(18.8)	300(300)	9.4(N)
<i>Listeria monocytogenes</i>	<sup>24</sup> MIC(MBC)	300(300)	300(600)	2400(2400)	-(-) <sup>d</sup>	18.8(37.5)	300(600)	37.5(N)
	<sup>48</sup> MIC(MBC)	600(600)	300(600)	2400(2400)	-(-)	18.8(37.5)	300(600)	37.5(N)
<i>Clostridium perfringens</i>	<sup>24</sup> MIC(MBC)	37.5(37.5)	150(150)	600(600)	<4800(<4800)	18.8(18.8)	75(300)	18.8(N)
	<sup>48</sup> MIC(MBC)	75(75)	150(150)	600(600)	<4800(<4800)	18.8(18.8)	150(300)	18.8(N)

<sup>a</sup> Minimum inhibitory concentrations and minimum bactericidal concentration (numbers in italic type in parentheses) after 24 hours of incubation

<sup>b</sup> Minimum inhibitory concentrations and minimum bactericidal concentration (numbers in italic type in parentheses) after 48 hours of incubation

<sup>c</sup> Not evaluated

<sup>d</sup> Not performed

**TABLE 3.** Minimum inhibitory concentrations and minimum bactericidal concentrations (mg.L<sup>-1</sup>) of natural substances determined for tested gram-negative after 24 and 48 hours

Microorganisms		Tested compounds						
		Carvacrol	Thymol	Eugenol	Gallic acid	Octyl gallate	Cnicin	Usnic acid
<i>Alcaligenes faecalis</i>	<sup>24</sup> MIC(MBC) <sup>a</sup>	300(300)	150(300)	600(600)	75(75)	75(75)	75(75)	2700(5400)
	<sup>48</sup> MIC(MBC) <sup>b</sup>	300(300)	150(300)	600(600)	75(75)	75(75)	75(75)	2700(5400)
<i>Escherichia coli</i>	<sup>24</sup> MIC(MBC)	75(75)	300(300)	1200(1200)	4800(4800)	300(300)	300(600)	5400(5400)
	<sup>48</sup> MIC(MBC)	75(75)	300(300)	1200(1200)	4800(4800)	600(600)	300(600)	5400(5400)
<i>Providencia stuartii</i>	<sup>24</sup> MIC(MBC)	600(600)	300(300)	2400(2400)	4800(4800)	150(150)	150(150)	1350(2700)
	<sup>48</sup> MIC(MBC)	600(600)	300(300)	2400(2400)	4800(4800)	150(150)	150(300)	2700(5400)
<i>Klebsiella pneumoniae</i>	<sup>24</sup> MIC(MBC)	2400(2400)	150(300)	600(600)	<4800(<4800)	1200(1200)	1200(1200)	>9600(>9600)
	<sup>48</sup> MIC(MBC)	2400(2400)	150(300)	600(600)	<4800(<4800)	1200(1200)	1200(1200)	>9600(>9600)
<i>Pseudomonas aeruginosa</i>	<sup>24</sup> MIC(MBC)	150(300)	600(1200)	1200(1200)	4800(4800)	1200(4800)	300(600)	>9600(>9600)
	<sup>48</sup> MIC(MBC)	300(300)	600(1200)	1200(1200)	4800(4800)	4800(4800)	300(300)	>9600(>9600)
<i>Bacteroides fragilis</i>	<sup>24</sup> MIC(MBC)	18.8(37.5)	37.5(75)	75(150)	4800(<4800)	9.4(18.8)	75(150)	9.4(N) <sup>c</sup>
	<sup>48</sup> MIC(MBC)	18.8(37.5)	75(150)	75(150)	4800(<4800)	18.8(18.8)	300(300)	18.8(N)

<sup>a</sup> Minimum inhibitory concentrations and minimum bactericidal concentration (numbers in italic type in parentheses) after 24 hours of incubation

<sup>b</sup> Minimum inhibitory concentrations and minimum bactericidal concentration (numbers in italic type in parentheses) after 48 hours of incubation

<sup>c</sup> Not evaluated

**TABLE 4.** Minimum inhibitory concentrations and minimum bactericidal concentrations (mg.L<sup>-1</sup>) of natural substances determined for tested *Candida* strains after 24 and 48 hours

Microorganisms		Tested compounds						
		Carvacrol	Thymol	Eugenol	Gallic acid	Octyl gallate	Cnicin	Usnic acid
<i>Candida albicans</i>	<sup>24</sup> MIC(MBC) <sup>a</sup>	150(150)	300(300)	1200(1200)	2400(2400)	18.8(18.8)	1200(2400)	>9600(>9600)
	<sup>48</sup> MIC(MBC) <sup>b</sup>	150(150)	300(300)	1200(1200)	2400(2400)	18.8(18.8)	1200(2400)	>9600(>9600)
<i>Candida catenulata</i>	<sup>24</sup> MIC(MBC)	75(75)	300(300)	600(600)	4800(<4800)	4.7(4.7)	1200(1200)	>9600(>9600)
	<sup>48</sup> MIC(MBC)	150(150)	300(300)	600(600)	<4800(<4800)	4.7(4.7)	1200(1200)	>9600(>9600)

<sup>a</sup> Minimum inhibitory concentrations and minimum bactericidal concentration (numbers in italic type in parentheses) after 24 hours of incubation<sup>b</sup> Minimum inhibitory concentrations and minimum bactericidal concentration (numbers in italic type in parentheses) after 48 hours of incubation

On the other hand, Tippayatum and Chonhenchob (2007) reported MIC values (3000 – 5000 mg.L<sup>-1</sup>) for thymol against *Listeria monocytogenes*, *S. aureus*, *Bacillus cereus* and *E. coli* that are approximately ten-fold higher than our results using the agar well dilution method.

The antimicrobial activity of eugenol against the microorganisms tested in our work was also considerable. Palaniappan and Holley (2010) reported that eugenol inhibited the growth of *E. coli* and *S. aureus* at a concentration of 410 mg.L<sup>-1</sup> using a microdilution method. The results of Medina et al. (2009) found no bactericidal activity for eugenol against *Pseudomonas fluorescens* and *Enterococcus faecalis* in the concentration range 82 820 mg.L<sup>-1</sup>. Our results for *Enterococcus* sp. and *P. aeruginosa* found MIC values of 1200 - 2400 mg.L<sup>-1</sup> and MBC values of 2400 mg.L<sup>-1</sup>. In our study, the lowest MIC and MBC values were determined against *Bacteroides fragilis* (MIC 75 mg.L<sup>-1</sup> and MBC 150 mg.L<sup>-1</sup>), see TABLE 3.

It has been previously reported that gallic acid is an effective antimicrobial compound exhibiting a 50% inhibition of *P. aeruginosa* growth at a concentration of 205 mg.L<sup>-1</sup> (Cueva et al., 2012). On the other hand, Binutu and Cordell (2000) found a higher MIC against *P. aeruginosa*, 1000 mg.L<sup>-1</sup>. The studies of the antibacterial effect of gallic acid (Al-Zahrani, 2012; Cueva et al., 2012) have reported a strong antibacterial activity of gallic acid against *S. aureus* strains, however streptococci appeared to be much more resistant than *S. aureus*. The antimicrobial activity of gallic acid could be associated with the presence of the 3,4,5-trihydroxyphenyl group (Cueva et al., 2012; Al-Zahrani, 2012).

We found gallic acid to have a strong antibacterial activity against *Alcaligenes faecalis* with an MIC of 75 mg.L<sup>-1</sup>. The clear inhibitory and bactericidal effects of gallic acid against most microorganisms were determined for concentrations of 2400-4800 mg.L<sup>-1</sup> or even higher (see TABLE 2,3,4). Since we used wide concentration ranges of the natural substances (see TABLE 1), we found that gallic acid is able to exhibit an inhibitory and bactericidal effect against *S. aureus*, *Providencia stuartii* and *P. aeruginosa* in the concentration range 75-1200 mg.L<sup>-1</sup> (not mentioned in TABLE 2 or 3). Nevertheless, at higher concentrations of gallic acid (2400-4800 mg.L<sup>-1</sup>), these bacteria were able to grow. One possible explanation may be supported by the ability of some bacteria to metabolize gallic acid. Alberto et al. (2004) reported that *Lactobacillus hilgardii* may produce catechol, p-hydroxybenzyl alcohol, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, protocatechuic acid and pyrogallol from gallic acid. These compounds may have stronger antibacterial effects than gallic acid. Another explanation may be based on the presence of the 3,4,5-trihydroxyphenyl group of gallic acid, which is prone to oxidation to form of semiquinones and other components (Eslami et al., 2010).

Octyl gallate inhibited all the examined microorganisms within the concentration range used. Octyl gallate was shown to have strong inhibitory activity against gram-positive bacteria, with MIC values of 18.8-75 mg.L<sup>-1</sup> and MBC values of 18.8-75 mg.L<sup>-1</sup>. Furthermore, the differences among the MICs and MBCs were no more than 2-fold, suggesting that the activity of octyl gallate is bactericidal. Against *Candida* strains, octyl gallate was the most effective substance tested, with MIC values of 4.7-18.8 mg.L<sup>-1</sup> and MFC values of 4.7-18.8 mg.L<sup>-1</sup> (see TABLE 4). Its MIC and MBC values against gram-negative bacteria were higher, especially against *P. aeruginosa* and *K. pneumoniae* (see TABLE 3). Similar results have been reported in the study by Kubo et al. (2001) and Kubo et al. (2002). These results suggest that octyl gallate is a potent substance for use in animal production with a broad antimicrobial spectrum.

The determination of the antimicrobial activity of cnicin revealed MIC values of 75-300 mg.L<sup>-1</sup> and MBC values of 75-600 mg.L<sup>-1</sup> for the tested microorganisms apart from the *K. pneumoniae* and *Candida* strains, therefore, we found cnicin to be highly potent against almost all the microorganisms examined. Similar results were reported by Mazurova et al. (2007) for *S. aureus*, *Enterococcus faecalis*, *P. aeruginosa* and *E. coli* using the macrodilution method in Mueller Hinton broth.

However, there is little information about the antimicrobial activity of cnicin in the literature. In contrast to the other tested natural substances, cnicin has a unique mechanism of action based on the inhibition of enolpyruvyl-transferase MurA, the bacterial enzyme involved in the first step in the cytoplasmic biosynthesis of the peptidoglycan precursor (Bugg et al., 2011; Steinbach et al., 2008). The mechanism of action of other natural substances is mainly based on their ability to disintegrate the cytoplasmic membrane of microorganisms. (Kalemba and Kunicka, 2003; Gill et Holley, 2004, Xu et al., 2008). In our study cnicin was obtained via extraction, because of its very high market price.

We found that usnic acid was the most potent natural substance, inhibiting the growth of all tested gram-positive bacteria with MIC values of 4.7-37.5 mg.L<sup>-1</sup>. Nevertheless, the differences among the MICs and MBCs were more

**TABLE 5.** Minimum inhibitory, bactericidal and fungicidal concentrations of control antimicrobial agents (mg.L<sup>-1</sup>) and ethanol (% v/v) determined for all tested microorganisms after 24 and 48 hours

Microorganisms		Tested antimicrobial agents			
		Ampicillin (mg.L <sup>-1</sup> )	Gentamicin (mg.L <sup>-1</sup> )	Amfotericin B (mg.L <sup>-1</sup> )	Ethanol (% v/v)
<i>Bacillus subtilis</i>	<sup>24</sup> MIC(MBC) <sup>a</sup>	0.06(0.06)	0.04(0.04)	-(-)	12(12)
	<sup>48</sup> MIC(MBC) <sup>b</sup>	0.06(0.06)	0.04(0.04)	-(-)	12(12)
<i>Enterococcus faecalis</i>	<sup>24</sup> MIC(MBC)	0.63(2.5)	10(20)	-(-)	12(24)
	<sup>48</sup> MIC(MBC)	2.5(2.5)	10(20)	-(-)	48(48)
<i>Staphylococcus aureus</i>	<sup>24</sup> MIC(MBC)	0.25(0.25)	0.31(0.31)	-(-)	6(6)
	<sup>48</sup> MIC(MBC)	0.25(0.25)	0.31(0.31)	-(-)	6(6)
<i>Streptococcus porcinus</i>	<sup>24</sup> MIC(MBC)	0.25(0.5)	4(4)	-(-)	3(6)
	<sup>48</sup> MIC(MBC)	0.25(0.5)	4(4)	-(-)	6(6)
<i>Listeria monocytogenes</i>	<sup>24</sup> MIC(MBC)	0.06(0.25)	0.25(0.25)	-(-)	3(24)
	<sup>48</sup> MIC(MBC)	0.125(0.25)	0.25(0.25)	-(-)	3(24)
<i>Clostridium perfringens</i>	<sup>24</sup> MIC(MBC)	-(-) <sup>c</sup>	-(-)	-(-)	12(24)
	<sup>48</sup> MIC(MBC)	-(-)	-(-)	-(-)	12(24)
<i>Alcaligenes faecalis</i>	<sup>24</sup> MIC(MBC)	10(10)	1.25(1.25)	-(-)	3(3)
	<sup>48</sup> MIC(MBC)	10(10)	1.25(1.25)	-(-)	3(3)
<i>Escherichia coli</i>	<sup>24</sup> MIC(MBC)	5(10)	1.25(1.25)	-(-)	6(12)
	<sup>48</sup> MIC(MBC)	5(10)	1.25(1.25)	-(-)	12(12)
<i>Providencia stuartii</i>	<sup>24</sup> MIC(MBC)	5(10)	10(10)	-(-)	6(6)
	<sup>48</sup> MIC(MBC)	10(10)	10(10)	-(-)	12(12)
<i>Klebsiella pneumoniae</i>	<sup>24</sup> MIC(MBC)	128(128)	0.125(0.125)	-(-)	6(6)
	<sup>48</sup> MIC(MBC)	256(256)	0.125(0.125)	-(-)	12(12)
<i>Pseudomonas aeruginosa</i>	<sup>24</sup> MIC(MBC)	256(512)	0.5(0.5)	-(-)	3(3)
	<sup>48</sup> MIC(MBC)	512(512)	0.5(0.5)	-(-)	3(3)
<i>Bacteroides fragilis</i>	<sup>24</sup> MIC(MBC)	-(-)	-(-)	-(-)	12(24)
	<sup>48</sup> MIC(MBC)	-(-)	-(-)	-(-)	12(24)
<i>Candida albicans</i>	<sup>24</sup> MIC(MBC)	-(-)	-(-)	0.5(0.5)	6(6)
	<sup>48</sup> MIC(MBC)	-(-)	-(-)	0.5(0.5)	6(6)
<i>Candida catenulata</i>	<sup>24</sup> MIC(MBC)	-(-)	-(-)	0.5(0.5)	3(3)
	<sup>48</sup> MIC(MBC)	-(-)	-(-)	0.5(0.5)	3(3)

<sup>a</sup> Minimum inhibitory concentrations and minimum bactericidal, for *Candida* strains fungicidal concentrations (numbers in italic type in parentheses) after 24 hours of incubation.

<sup>b</sup> Minimum inhibitory concentrations and minimum bactericidal, for *Candida* strains fungicidal concentrations (numbers in italic type in parentheses) after 48 hours of incubation.

<sup>c</sup> Not performed



than 2-fold, suggesting that the activity of usnic acid is not bactericidal. Usnic acid was virtually ineffective Against gram-negative bacteria and *Candida* strains, or only effective at high concentrations (see TABLE 3 and 4). Lauterwein et al. (1995) reported similar results for the susceptibility of *S. aureus* and *Enterococcus faecalis* to usnic acid. Rankovic et al. (2008) evaluated the antibacterial activity of usnic acid isolated from plant *Parmelia caperata* with the same results for *S. aureus* and similar results for *Bacillus subtilis*. The same authors found the growth of 3 members of Enterobacterales (*E. coli*, *K. pneumoniae*, *Enterobacter cloacae*) were inhibited with usnic acid in the concentration range of 3.7-31 mg.L<sup>-1</sup>, whereas Lauterwein et al. (1995) showed that usnic acid did not inhibit the growth of *E. coli*, *P. aeruginosa* and *Candida albicans* at a concentration of 32 mg.L<sup>-1</sup>. In our study, the observed MICs for gram-negative bacteria were higher (1350-9600 mg.L<sup>-1</sup>). The MICs for *P. aeruginosa* and *K. pneumoniae* were even more than 9600 mg.L<sup>-1</sup>. Interestingly, pharmacokinetic studies in rabbits have shown that usnic acid is well absorbed after oral administration, supporting its possible use in animal rearing (Krishna and Venkataramana, 1992).

Discrepancies in the results might be caused by using a different solvent or different method for the determination of antimicrobial activity. We used EtOH as the solvent with low toxicity, other authors used DMSO (Rankovic et al., 2008), acetone extract (Tay et al., 2004) or tetrahydrofuran (Lauterwein et al., 1995). In addition, EtOH was not found to have antimicrobial effects in used concentration (1%, v/v). *E. faecalis* was able to survive and grow even in 48% ethanol (see Table 5).

## CONCLUSION

In summary, each of the tested substances exhibited antimicrobial activity against some of the tested microorganisms. Thymol, carvacrol, cnicin, eugenol and octyl gallate were found to exhibit inhibitory and bactericidal activities against all the tested microorganisms in the concentration ranges used, exhibiting a broad antimicrobial spectrum. Our results suggest the possible involvement of the evaluated natural substances in the difficult process of animal breeding with respect to the lower toxicity and good availability of the majority of the examined substances. Further investigation in this field should be focused on combinations of natural substances in order to extend their spectrum of antimicrobial efficacy or to determine possible synergistic effects of combinations of them.

## Funding

This work was supported by the Ministry of Agriculture of the Czech Republic (Project No. QI111A166)

## Conflict of interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

## Adherence to ethical standards

This article does not contain any studies involving animals performed by any of the authors.

This article does not contain any studies involving human participants performed by any of the authors.

## Authors' contributions

R. Kukla: made substantial contributions to conception and design, acquisition of data regarding the MIC and MBC of natural substances, analysis and interpretation of data, mainly involved in drafting the manuscript, given final approval of the version to be published. J. Mazurova: made substantial contributions to acquisition of data and interpretation of data, involved in drafting the manuscript. I. Krovakova: made substantial contributions to performing the assays for MIC and MBC determination. E. Slehova: agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. R. Sleha: agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. M. Rozkot: agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. L. Opletal: made substantial contributions to extraction of cnicin.

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