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ORIGINAL ARTICLE

IN VITRO ANTIBACTERIAL ACTIVITY OF USNIC ACID AND OCTYL GALLATE AGAINST RESISTANT *ENTEROCOCCUS* STRAINS

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Summary

The genus *Enterococcus* is represented by some of the clinically important species and is well known for its antibiotic resistance, which participates in colonization and infection. Increasing resistance of enterococci has been evoked due to the excessive use of antibiotics not merely for therapeutic purposes or in indicated cases. One of the promising possibilities how to reduce the use of great amount of antibiotics is to utilize antimicrobial properties of natural substances. Usnic acid is a lichen compound possessing antibacterial activity against gram-positive bacteria including enterococci. Octyl gallate, gallic acid ester, has significant antibacterial and antifungal properties. This study was focused on the evaluation of resistant enterococci susceptibility to usnic acid and octyl gallate in comparison with control group of enterococci. Antibacterial activity of usnic acid and octyl gallate was defined as minimum inhibitory concentration (MIC value) and minimum bactericidal concentration (MBC value). Usnic acid inhibited all tested enterococci in concentration range 4.7-37.5 mg.L^{-1}. MIC values of octyl gallate for all tested enterococci ranged between 37.5-150 mg.L^{-1}. In contrast to octyl gallate, bactericidal activity of usnic acid was not confirmed. Antibacterial activities of tested compounds were almost equal among resistant enterococci and control group of enterococci exhibiting great potential of usnic acid and octyl gallate for treatment of enterococcal infections.

Key words: enterococci; octyl gallate; usnic acid; MIC; MBC

INTRODUCTION

Enterococci are commonly recognized as part of the natural microflora in the gastrointestinal tract also occurring as ubiquitous inhabitants in diverse environments [1,2]. These bacteria possess an increased ability to survive under a wide range of adverse conditions such as lack of moisture, extreme temperature and high osmolality [2,3]. During the last decades, enterococci have emerged as serious nosocomial pathogens, responsible for bloodstream infections, endocarditis, post-operative wounds, skin and soft tissue infections, urinary tract infections and rarely meningitis [2,4,5]. According to ECDC 2013 (European Centre for Disease Prevention and Control), enterococci were the second and the third most

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frequently isolated microorganisms from bloodstream infections and healthcare-associated infections, respectively [6].

The clinical significance of enterococci is also associated with a high rate of their antibiotic resistance. Enterococci are naturally resistant to the majority of cephalosporins and semi-synthetic penicillins (due to their expression of low-affinity penicillin-binding proteins), aminoglycosides (low level resistance), clindamycin and have a decreased susceptibility to penicillin and ampicillin [7]. In addition, the acquired resistance may also occur in enterococci through sporadic mutations or horizontal exchange of genes encoding resistance determinants [5]. Consequently, enterococci may exhibit high level resistance to penicillin and aminoglycosides, furthermore, resistance to glycopeptides and rarely to linezolid, daptomycin and tigecycline may also occur [5,8].

The most important species for humans are *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) [4]. Although the majority of infections are still caused by *E. faecalis*, the numbers of *E. faecium* infections have recently increased in the USA and Europe [2]. *E. faecalis* is considered to express more virulence factors than *E. faecium*, whereas *E. faecium* is known to possess a higher level of antibiotic resistance in comparison to *E. faecalis* [2,9]. Nevertheless, other species involving *E. durans*, *E. casseliflavus*, *E. gallinarum* and *E. hirae* are occasionally isolated from clinical material [9-11].

It is obvious that the increase in numbers of microorganisms resistant to the antimicrobial agents including enterococci especially due to the high selective antibiotic pressure has been a global challenge. It is essential to reduce and restrict prescription of antibiotics and to search for new possibilities of antimicrobial treatment. In order to decrease the use of antibiotics, a convenient alternative might be to utilize natural substances or their derivatives, such as lichen compounds, gallates or phenolic compounds with known antibacterial activity [12-14]. In this study, antibacterial activities of octyl gallate and usnic acid were determined.

Octyl gallate is a member of alkyl gallates group, which are derivatives of naturally occurring compound gallic acid [15]. Octyl gallate is considered to be a synthetic ester of gallic acid, however this compound has also occurred in fruits of *Terminalia bellerica* as it was recently proven by Latha and Daisy 2013 [16]. Octyl gallate is almost insoluble in water, 14 mg.L-1 at 25°C [17], freely soluble in ethanol [18], methanol [19], ether and in different class of emulsifiers [20].

This compound is primarily known for pronounced antioxidant properties, which were firstly reported in the late forties of the 20th century by Morris et al. 1947 [21]. Octyl gallate is used as the antioxidant due to its ability to reduce the oxidation of unsaturated fats by the food industry in the United States (US FDA, updated on 11th November, 2011) and is also included in the European Union list of food additives approved for the use in foods [22-24]. In Japan, octyl gallate is recognized as quasi drug by the Ministry of Health [25]. Unlike other synthetic antioxidants, octyl gallate has not been shown as possible cancer precursor [26].

It has to be emphasized that octyl gallate possesses significant antifungal activity and also strong antibacterial effects especially against gram-positive bacteria [18,24,27,28]. In addition, antiviral effects against DNA as well as RNA viruses were established [25]. Furthermore, some studies mentioned that octyl gallate and other gallic acid derivatives are selectively cytotoxic to tumor cells [29,30]. Unfortunately, available studies about toxicological properties of octyl gallate are limited. Mechanisms of antibacterial and antifungal action are associated with a balance between the hydrophobicity of the side chain and the hydrophilicity by hydroxyls on the benzene ring and with the ability to inhibit the membrane respiratory systems. Octyl gallate acts as nonionic surfactant and it is able to inhibit efflux pumps in case of *Saccharomyces cerevisiae* [31-33].

Usnic acid is a dibenzofuran derivative widely occurring as a secondary metabolite in lichens. It was firstly isolated by Knop in 1844, cited by Ingólfsdóttir 2002 [34]. Species *Cladonia*, *Usnea*, *Alectoria*, *Parmelia* and many others are well known lichens in which usnic acid is present [34-36]. In slow-growing lichens, the role of usnic acid is presumably to protect against pathogens [37]. Usnic acid is poor water soluble, less than 100 mg.L^{-1} at 25°C [38,39]. Solubility in organic solvents is in the descending order ethyl acetate > acetone > n-hexane > ethanol [40].

This compound has been intensively studied since the 1950s, particularly for its antimicrobial activity [41]. Usnic acid has been used as a pure

substance in creams, toothpastes, deodorants etc., either as an active compound or preservative [42]. In the United States, usnic acid has been available as a dietary supplement to aid in weight loss [43]. Nevertheless, many studies have shown adverse effects of usnic acid, namely its hepatotoxicity due to its ability to inhibit and uncouple oxidative phosphorylation in hepatocytes [44,45]. Local irritation and allergic contact dermatitis may occur rarely [34]. As it has been shown in many previous studies, usnic acid possesses strong antibacterial activity, especially against gram-positive bacteria [13,37,41,46]. It is also effective against anaerobic bacteria, mycobacteria, some yeasts and fungi [41,47]. Mechanism of antibacterial action of usnic acid is probably based in its ability to disrupt bacterial cell membrane [48].

The aim of this study was to evaluate the minimum inhibitory concentrations (MIC values) and minimum bactericidal concentration (MBC values) of compounds mentioned above by using a standardized microdilution method against resistant and control group of enterococci to see whether there were differences in antibacterial activity among resistant and control group strains.

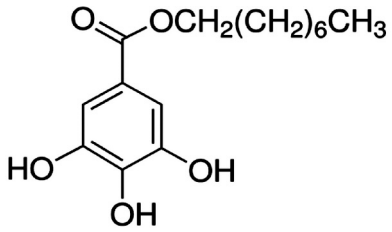


Figure 1. Octyl gallate - Octyl 3,4,5-trihydroxybenzoate

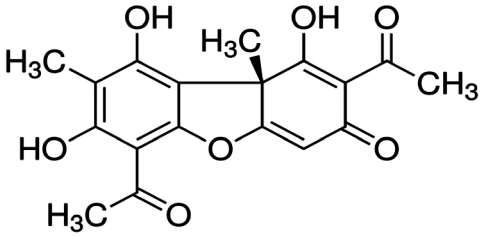


Figure 2. (+)-usnic acid - (9bS)-2,6-Diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione

MATERIALS AND METHODS

Microbial strains

Microbial strains used as test organisms (a total of 19) excluding reference strain *Enterococccus faealis* CCM 4224 from the Czech Collection of Microorganisms were isolated from patients suffering from diseases associated with local inflammation in General University Hospital in Prague. Antibiotic susceptibility testing was performed by the disk diffusion method, with Mueller-Hinton agar according to EUCAST. Enterococci were divided into the group of resistant enterococci, (n-number of strains=12) and the control group of enterococci (n=7). The group of resistant enterococci included *E. faecium* (n=9), *E. faecalis* (n=2) and *E. gallinarum* (n=1). The control group consists of *E. faecium* (n=1), *E. faecalis* (n=4, reference strain included) and *E. durans* (n=2).

Enterococci were maintained on blood agar plates (Hi Media, India) with 5% of sheep defibrinated blood and cultures were stored at 4°C and subcultured once a month if necessary.

Culture media

Cation adjusted Mueller-Hinton broth (Hi Media, India) was used for determination of minimum inhibitory concentrations. Blood agar plates with 5% of sterile sheep defibrinated blood were used for determination of minimum bactericidal concentration.

Natural substances and antimicrobials

Octyl gallate was purchased from Sigma-Aldrich (St. Louis, MO, USA). (+)-Usnic acid was purchased from Carl-Roth (Germany). Antimicrobial agent Ampicillin was received from Biotika (Slovakia).

Antimicrobial assay

For the experiment, calculated amount of natural compound was first dissolved in a small amount of 96% ethanol. After the dissolution of substance, calculated amount of broth was added. Final concentration of ethanol in stock solution did not exceed 1.0% (vol/vol) in the experiment. The suitable ranges of natural substances concentrations used for determination of susceptibility were prepared in two-fold dilution steps.

Minimum inhibitory concentration (MIC value) was determined by the microdilution method, inhibitions or growths of microorganisms were evaluated visually. The bacterial inocula prepared from freshly subcultivated 24 hours cultures were standardized with nephelometer (Erba Lachema, CZ) and subsequently diluted in sterile physiologic saline. Density of bacterial suspension after application to wells of microtiter plates with natural substances responded to yield approximately $0.5x10^6$ CFU.mL^{-1}. 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 under aerobic conditions.

The MIC value was defined as the lowest concentration in the wells of the microtiter plate that showed no turbidity, i.e. 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 showing no growth of enterococci. The content of corresponding wells was inoculated with use of 1 µl calibrated bacterial loop on sterile blood agar plate. The MBC value was defined as the lowest concentration of natural substance, that caused a ≥ 99.9% reduction from that original inoculum, i.e., the first concentration without observed growth of enterococcal colonies after 24 hours of incubation at 37°C.

Determination of antimicrobial activity of each substance was performed in triplicates. MIC and MBC values were presented as median values. Growth controls and sterility of medium controls were performed simultaneously with determination of antimicrobial activity. The growth control was broth containing 1.0% ethanol (vol/vol) where the corresponding microbial suspension was pipetted. Sterility of medium control was sterile Mueller-Hinton broth undergoing the incubation conditions mentioned above.

RESULTS AND DISCUSSION

We have investigated antibacterial activity of usnic acid and octyl gallate against enterococci. Enterococci were divided into the group of resistant enterococci and the control group of enterococci. Enterococci in the first group were resistant at least to one of previously tested antibiotics (ampicillin, furazolidone, vancomycin, teicoplanin, linezolid, gentamicin), as determined by a disk diffusion method.

Table 1. MIC and MBC values (mg.L^{-1}) of usnic acid, octyl gallate and antibiotic ampicillin against resistant enterococci evaluated after 24 and 48 hours

		Usnic acid		Octyl gallate		Ampicillin	
Microorganisms	t	MIC	MBC	MIC	MBC	MIC	MBC
Enterococcus faecium (n=9)	24	4.7-9.4	N	75	75	>32	>32
	48	9.4-18.8	N	75-150	75-150	>32	>32
Enterococcus faecalis (n =2)	24	9.4-18.8	N	37.5-75	75	N	N
	48	9.4-18.8	N	75-150	75-150	N	N
Enterococcus gallinarum (n =1)	24	18.8	N	75	75	2	4
	48	18.8	N	75	75	4	8

t-incubation time in hours, N-not evaluated

Table 2. MIC and MBC values (mg.L^{-1}) of usnic acid, octyl gallate and antibiotic ampicillin against control group of enterococci evaluated after 24 and 48 hours

		Usnic acid		Octyl gallate		Ampicillin	
Microorganisms	t	MIC	MBC	MIC	MBC	MIC	MBC
Enterococcus faecium (n=1)	24	9.4	N	75	75	2	4
	48	9.4	N	75	75	4	4
Enterococcus faecalis (n =4)	24	9.4	N	37.5-75	75	1-2	1-2
	48	9.4-18.8	N	75	75	1-2	1-2
Enterococcus durans (n =2)	24	37.5	N	75	75	2-4	2-4
	48	37.5	N	75	75	2-4	2-4

t-incubation time in hours, N-not evaluated

Enterococci in the second group were susceptible to these antibiotics. The examined ranges of concentrations for both usnic acid and octyl gallate were 1.2-600 mg.L^{-1}. For the investigation of antibacterial activity of natural substances, we used a microdilution method because it represents frequently used methodology which can be easily standardized for determination MIC values. Furthermore, MBC values were evaluated after subculture of wells without visible growth of bacteria. Since ethanol is a suitable solvent with a little direct effect on human cells, we used it for dissolution of natural substances [38].

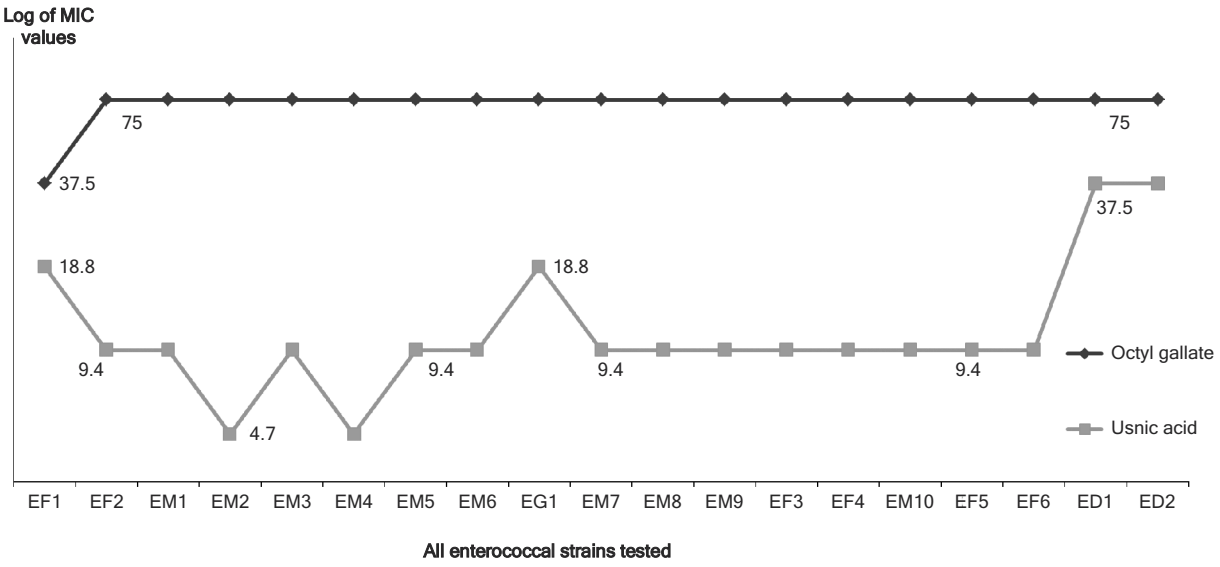
Octyl gallate is a poorly water soluble compound (14 mg.L^{-1} at 25°C) [17], therefore, we ensured proper solubilization by dissolving it in a small amount of ethanol. Subsequently, dissolution was supported by adding MHB warmed up to 37°C, because as reported by Lu et al. 2007 [17], the solubility of octyl gallate is directly proportional to the temperature.

We found octyl gallate to exhibit inhibitory and bactericidal activity against all examined strains of enterococci within the range of concentrations used. We determined that MIC values of octyl gallate for all resistant enterococci were 75 mg.L^{-1} after one day of incubation as listed in Table 1. MBC values of octyl gallate for the majority of control group enterococci were also 75 mg.L^{-1} (Table 1,2). Little differences in MIC and MBC values using subsequent higher dilution of octyl gallate were apparent after two days of incubation. Interestingly, we ascertained MIC and MBC values after 3 days of incubation (data not shown), which were identical to the results after two days of incubation. Furthermore, the differences among the MIC and MBC values were not more than 2-fold, suggesting that activity of octyl gallate against enterococci is bactericidal. To sum up, we did not find any significant differences in the susceptibility to octyl gallate between the enterococci resistant or susceptible to antibiotics.

Our results confirm pronounced effects of octyl gallate on gram-positive bacteria. Gutiérrez-Larraínzar et al. 2013 [24] showed better antibacterial activity of octyl gallate in comparison to propyl gallate against *Staphylococcus aureus* strains with the range of MIC values of 6.25-29.17 mg.L^{-1}. Kubo et al. 2001 [27] determined that octyl gallate is more effective than propyl gallate against *Micrococcus luteus*, *Streptococcus mutans*, *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* strains with MIC values 12.5 mg.L^{-1}, 50 mg.L^{-1}, 25 mg.L^{-1}, 25 mg.L^{-1}, respectively.

To our knowledge, there is no previously published data concerning the antibacterial activity of octyl gallate against resistant enterococci compared to the control group of enterococci and these have been determined and presented in this study. From our and published results, it is evident that octyl gallate has a broad antimicrobial spectrum against gram-positive bacteria. Many published data also showed significant antifungal activity of this compound against different species of yeasts and fungi [18,27,28,49]. In addition, Törmäkangas et al. 2005 [15] reported an inhibitory effect of octyl gallate against *Chlamydia pneumoniae* in mice with lung infection treated with this compound.

Possible application of octyl gallate in treatment may be limited due to its decreased enteral absorption, as only 20-30% of octyl gallate was detected in tissues after oral administration to rats.



EF – *E. faecalis*, EM – *E. faecium*, EG – *E. gallinarum*, ED – *E. durans*

Figure 3. Distribution of MIC values (mg.L^{-1}) of octyl gallate and usnic acid against all enterococci tested after 24 hours of incubation

However up to 12 hours after administration, 60-80% of octyl gallate was found in gastro-intestinal tract [50]. Furthermore, the relative low contact allergy reactions to 0.3% octyl gallate developed in patients with chronic leg ulcers in comparison with antiseptics cetrimide (0.1%) or thimerosal (0.1%) [51].

Usnic acid has been proven to have antibacterial activity against a broad spectrum of gram-positive bacteria including resistant clinical isolates of enterococci and *Staphylococcus aureus* [13,37,46]. Usnic acid is the most effective compound against mycobacteria in comparison with the other lichen metabolites [41]. Lucariny et al. 2012 [52] have reported the activity of usnic acid against reference strains of *Mycobacterium tuberculosis*, *Mycobacterium kansasii* and *Mycobacterium avium* with MIC values 8 mg.L^{-1}, 8 mg.L^{-1}, 16 mg.L^{-1}, respectively. In addition, usnic acid has been found to possess potent inhibitory activity against *Candida parapsilosis* and *Candida orthopsilosis* [53] and against several other fungi [54]. Furthermore, its use as an adjuvant therapy of Human Papillomavirus genital infection reduced the recurrence and improved the time of reepithalization [55].

We have found that usnic acid inhibited growth of enterococci in lower concentrations in comparison with octyl gallate, (MIC values 4.7-37.5 mg.L^{-1}). Our results are comparable with those reported by Lauterwein et al. 1995 [56] who determined MIC values 4-8 mg.L^{-1} and 4-16 mg.L^{-1} of (+) enantiomer of usnic acid for *E. faecalis* and *E. faecium* after 24 hours of incubation, respectively. Elo et al. 2007 [13] showed MIC values 7.8-16 mg.L^{-1} and 3.9-7.8 mg.L^{-1} for vancomycin-resistant *E. faecalis* and *E. faecium* strains after 24 hours of incubation, respectively.

Nevertheless, we found that the differences among the MIC and MBC values were more than 2-fold, suggesting that the activity of usnic acid is not bactericidal as it was seen in the case of octyl gallate. Our results show the ability of usnic acid to inhibit the growth and multiplication of enterococci very effectively in low concentrations, however, the limited number of bacteria are probably capable of surviving even in the highest concentration tested. We have performed the determination of MIC and MBC values of usnic acid three times in triplicates in order to confirm these results. The effect of usnic acid is more likely bacteriostatic, which is in accordance with the study of Lauterwein et al. 1995 [56].

In this study, we did not find any significant differences in MIC values among resistant enterococci and control group of enterococci. According to the MIC values obtained for enterococci tested, usnic acid demonstrated potent inhibitory activity in relatively low concentrations. It indicates possible involvement of usnic acid in the treatment of enterococcal infections, which may be supported by its good absorption after administration [57].

On the other side, the use of usnic acid, particularly (+)-usnic acid may be limited due to its potential hepatotoxicity [44,45]. However, the other option is to exploit antimicrobial activity of (-) enantiomer of usnic acid which was found to be safe up to 100 mg/kg body weight [58] with almost the same antibacterial activity as (+) enantiomer of usnic acid [56]. In addition, the examination of interaction between usnic acid and currently licensed antibiotics may be convenient to overcome antibiotic-mediated resistance [59].

CONCLUSION

An increase in antibiotic resistance of bacteria even to the most recently approved antibiotics has been a world-wide problem. There is a significant need for the development of new antibiotics or for revitalizing the already used ones. Nevertheless, such options are currently almost exhausted mainly because of the excessive use of antibiotics. One of the possible means to find new opportunities is to investigate antimicrobial activity of potent substances derived from plants or living wild organisms including lichens.

The data obtained in this study show notable results of antibacterial activity of usnic acid and octyl gallate against enterococcal strains and suggest the potential application of these substances in the treatment of enterococcal infections. Nevertheles, further investigations in this field including toxicity evaluation, pharmacokinetics after administration or derivatization of these compounds should be performed in order to reach clinical trials and to allow further applications.

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