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Strong antimicrobial activity of xanthohumol and other derivatives from hops (*Humulus lupulus* L.) on gut anaerobic bacteria

PAVEL CERMAK,[1] JANA OLSOVSKA,[2] ALEXANDR MIKYSKA,[2] MARTIN DUSEK,[2] ZUZANA KADLECKOVA,[1] JIRI VANICEK,[3] OTAKAR NYC,[4] KAREL SIGLER,[5] VANDA BOSTIKOVA[6] and PAVEL BOSTIK[6]

[1]Thomayer Hospital, Videnska 800, Prague; [2]Research Institute of Brewing and Malting, PLC, Prague; [3]Faculty of Pharmacy, Charles University, Hradec Králové; [4]Department of Medical Microbiology 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, Prague 5; [5]Institute of Microbiology, Czech Academy of Sciences, Prague 4; and [6]Faculty of Military Medical Sciences, University of Defence, Hradec Kralove, Czech Republic

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Anaerobic bacteria, such as *Bacteroides fragilis* or *Clostridium perfringens,* are part of indigenous human flora. However, *Clostridium difficile* represents also an important causative agent of nosocomial infectious antibiotic-associated diarrhoea. Treatment of *C. difficile* infection is problematic, making it imperative to search for new compounds with antimicrobial properties. Hops (*Humulus lupulus* L.) contain substances with antibacterial properties. We tested antimicrobial activity of purified hop constituents humulone, lupulone and xanthohumol against anaerobic bacteria. The antimicrobial activity was established against *B. fragilis*, *C. perfringens* and *C. difficile* strains according to standard testing protocols (CLSI, EUCAST), and the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBC) were calculated. All *C. difficile* strains were toxigenic and clinically relevant, as they were isolated from patients with diarrhoea. Strongest antimicrobial effects were observed with xanthohumol showing MIC and MBC values of 15–107 μg/mL, which are close to those of conventional antibiotics in the strains of bacteria with increased resistance. Slightly higher MIC and MBC values were obtained with lupulone followed by higher values of humulone. Our study, thus, shows a potential of purified hop compounds, especially xanthohumol, as alternatives for treatment of infections caused by select anaerobic bacteria, namely nosocomial diarrhoea caused by resistant strains.

Key words: Xanthohumol; gut; anaerobic bacteria; *Clostridium*.

Pavel Bostik, Faculty of Military Health Sciences, University of Defence, Trebesska 1575, Hradec Kralove 50001, Czech Republic. e-mail: pavel.bostik@unob.cz

Antibiotics represent fundamental tools in modern medicine. However, their range of use is declining under the pressure of growing bacterial resistance in both community and, particularly, hospital settings. The need for new antibiotic compounds is supported by many reports on spreading antimicrobial resistance that is, unfortunately, faster than our ability to fill the arising therapy voids (1).

Since the creation of a new antibiotic is a lengthy and expensive process with an uncertain outcome and the ability of bacteria to develop resistance is unpredictable in its occurrence, the discovery and development of new antimicrobial agents are becoming a global challenge. The current, mainly synthetic approaches to create new antimicrobials can be expanded by utilizing natural resources. One source of antimicrobial substances can be the traditional herbal medicines that have been used since ancient times. As such, the hop plant (*Humulus lupulus* L.) with its long history in traditional medicine was used as the therapy against plentiful conditions including bacterial infections and also for its preservative qualities (2).

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Antibacterial properties of hop compounds are well known and have been applied in beer production since 11th century, in particular against beer spoiling bacterial strains (3). Since then, antibacterial activities against other bacteria, activities against some viruses, fungi and protozoa have been reported (4, 5). For example, in his 2015 study, Evrendilek described an effect of hop essential oil on pathogenic bacteria such as *Yersinia enterocolitica*, *Salmonella enteritidis* and *S. typhimurium*, *Proteus mirabilis*, *Escherichia coli* O157:H7 and *Klebsiella oxytoca* (4).

Testing of antimicrobial properties of hop compounds is mainly performed *in vitro*, while *in vivo* tests are rather sparse. There are reports describing their use in oro-dental infections because of their activity against oro-dental pathogens (6). Hop plant extracts were also tested for their activities against bacterial strains participating in acne vulgaris and strong antimicrobial effects of xanthohumol, and lupulones were proven against *Propionibacterium acnes, Staphylococcus epidermidis, S. aureus, Streptococcus pyogenes* and *Kocuria rhizophila*. Moreover, anticollagenase and antioxidant properties of hop components were also demonstrated (7).

Anaerobic bacteria represent part of human gut microbiome, but in certain conditions can cause serious health-affecting complications. Anaerobic infections (often caused by *Clostridium* sp. or/and *Bacteroides* sp. strains) are frequent complications in abdominal surgery, in gut lesions or obstetric medicine. They are very often diagnosed in mixed infections with facultative aerobic bacteria, such as enterobacteria, enterococci. *Clostridium difficile* is the most important cause of hospital-acquired diarrhoea, and the incidence of *C. difficile* infections (CDI) has an increasing trend. A severe course of the disease, increased mortality and hospital CDI outbreaks are associated with worldwide spread of PCR ribotype 027 (8).

The number of studies showing effects of hop extracts against anaerobic bacteria is rather low. Hop compounds have been shown to significantly reduce the *Clostridium* population, including *Clostridium perfringens* and *Bacteroides* population in broiler chickens (9). However, in such studies, either indirect methods or quantitative PCR assays to detect the amount of bacteria in animal gut were used to demonstrate an inhibitory effect of lupulone *in vivo*, which do not lead to the estimation of MIC/MBC values of the compounds under study.

The aim of our study was thus to determine the antibacterial properties (MIC/MBC) of hop extracts of α-bitter acids (a mixture of homologues), β-bitter acids (a mixture of homologues) and xanthohumol against *C. difficile*, *C. perfringens* and *B. fragilis*.

MATERIALS AND METHODS

Hop extracts

Four isolates of hop components were prepared for microbiological tests.

Pure isolates of α- and β-bitter acids were prepared at the Hop Research Institute in Zatec according to the procedure described by Krofta et al. (10). The starting material was CO_2 hop extract. The first step involved the partitioning of the extract solution in an alkaline medium of sodium carbonate and sodium hydroxide to separate the α- and β-acids fractions. The beta fraction was used in the next step for the preparation of pure β-acids (purity 99.7%) isolated through crystallization from the solvent mixture. The alpha fraction was used for the preparation of α-acids with a purity of 93% (the remainder consisted of unspecified resins).

The fourth preparation was a pure isolate of xanthohumol (95% w/w) obtained from the Hopsteiner research laboratory.

Bacterial strains

The antimicrobial effects of isolated hop extracts were tested on the strains of *Bacteroides fragilis* (n = 7), including metronidazole/clindamycin-resistant strain (MTR/CLI), *Clostridium perfringens* (n = 5) from the culture collection of Department of Microbiology, Thomayer Hospital in Prague, and *Clostridium difficile* (n = 28) from the culture collection of the Institute of Microbiology, University Hospital and 2nd Medical faculty, Charles University in Prague. Automatic systems Api (bioMerieux, Grenoble, France) and MALDI-TOF Biotyper (Bruker Daltonics, Germany) were used for identification.

Clostridium difficile strains were characterized using PCR ribotyping and detection of the presence of toxin production governing genes (tcdA (A), tcdB (B), cdtA and cdtB (binary)) by a multiplex PCR (11).

Evaluation of MIC and minimum bactericidal/bacteriostatic concentration (MBC/MBS) of hop extracts

The dried hop extracts were appropriately stored and were reconstituted in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Prague, Czech Republic) before analysis. For each experiment, fresh batch of tested samples was reconstituted.

The bacteria were grown for 48 h at 35 °C in an anaerobic workstation (BUGBOX, Ruskinn, Bridgend, UK) on Schaedler blood agar (Oxoid Ltd., Basingstoke, UK) and microbial suspensions (10^6 CFU/mL) were prepared in 5 mL of buffer saline (TRIOS, Prague, Czech Republic). The stock solutions of hop extracts were diluted in cultivation broth (Schaedler broth, Oxoid Ltd.) in glass tubes using twofold dilution system so that the concentration of the compounds ranged from 0.002 to 2 mg/mL. All cultivation broths in tubes were regenerated (20-min boiling in water bath and quick cooling down to room temperature). The tubes were then inoculated with microbial suspension of tested bacterial strains. Tubes inoculated with tested bacterial strain but without hop extracts were used as

positive control, and tubes containing tested hop extracts only without bacterial strains were used as negative control. The tubes were incubated for 48 h at 35 °C in anaerobic workstation, and MICs were then determined as the lowest concentration of the extracts that visibly inhibited microbial growth. To determine bactericidal concentration and to control the growth of anaerobic strains, all the tubes were inoculated on Schaedler blood agar and on Columbia blood agar (BIO RAD, Marnes-la Coquette, FRANCE). Schaedler blood agar plates were incubated for the subsequent 48 h at 35 °C in anaerobic workstation, and Columbia blood agar plates were incubated for the subsequent 24 h at 35 °C/5%CO_2. Either negative or positive growth of microbial colonies then determined the bactericidal or bacteriostatic concentration of hop extracts, respectively.

MIC was determined as the lowest concentration of the tested substance that visibly inhibits the growth of the bacterial strain.

Statistical evaluation

Statistical evaluation of the results of analysis of variance was performed using QC-Expert software version 3.1.

RESULTS AND DISCUSSION

The standardized assays led to the identification of MIC of α-bitter acids (a mixture of homologues), β-bitter acids (a mixture of homologues) and xanthohumol as described in Section 'Evaluation of MIC and minimum bactericidal/bacteriostatic concentration (MBC/MBS) of hop extracts'.

The results obtained with seven strains of *Bacteroides fragilis* and five strains of *Clostridium perfringens* are presented in Table 1. The MIC of α-bitter acids (a mixture of homologues) ranged from 160 μg/mL to 1.54 mg/mL for *B. fragilis* strains and from 680 μg/mL to 1.37 mg/mL for *C. perfringens* strains. Slightly better antibacterial effects were observed in case of β-bitter acids, with MICs ranging from 50 μg/mL to 430 μg/mL for *B. fragilis* strains and from 150 μg/mL to 250 μg/mL for *C. perfringens* strains. However, the best antibacterial effects were obtained with xanthohumol, where the MICs were approximately 10 times lower (MICs ranged from 15 to 60 μg/mL for *B. fragilis* strains and from 10 μg/mL to 50 μg/mL for *C. perfringens* strains) compared with α- and β-bitter acids.

The antibacterial assays with *C. difficile* were performed with clinically relevant toxigenic strains (positive tests for toxin A and toxin B production), which were isolated from patients with clinical symptoms of CDI diarrhoea (Table 2). The MIC of α-bitter acids ranged from 320 μg/mL to 2.04 mg/mL, while the MICs of β-bitter acids and xanthohumol were, in this case, better and in similar ranges (from 9 to 80 μg/mL and from 32 to 107 μg/mL, respectively).

Stronger antibacterial effects of β-bitter acids (lupulones) compared with α-bitter acids (humulones) were also detected in aerobic bacterial strains by others (7). The course of inhibitory effects of α-bitter and β-bitter acids was different from that of xanthohumol. Effects of bitter acids at four consecutive rising concentrations before the break point concentration were gradually increasing from turbidity to the reduced sediment. Unlike β-bitter acids, xanthohumol showed a sharp break point from turbidity to total growth inhibition at the concentration following the break point. These observations probably reflect a difference between the mechanisms of action of bitter acids and xanthohumol. The antimicrobial effect of bitter acids is not

Table 1. Antibacterial activities (MIC and MBC) of α-bitter acids (a mixture of homologues), β-bitter acids (a mixture of homologues) and xanthohumol against anaerobic strains *Bacteroides fragilis* and *Clostridium perfringens* determined by dilution broth assay (MIC determination) followed by growth on solid media (MBC determination)

	Compound mg/mL					
	α-bitter acids		β-bitter acids		Xanthohumol	
	MIC[1]	MBC[2]	MIC[1]	MBC[2]	MIC[1]	MBC[2]
B. fragilis 1	0.16	0.35	0.19	0.45	0.015	0.024
B. fragilis 4	0.68	1.37	0.34	0.77	0.056	0.080
B. fragilis 11	0.90	1.79	0.32	0.58	0.029	0.029
B. fragilis 24	1.54	2.05	0.43	0.85	0.048	0.056
B. fragilis 26	1.15	1.54	0.26	0.85	0.044	0.052
B. fragilis 35	0.26	0.51	0.22	0.45	0.028	0.040
B. fragilis 489 MTR/CLI -	0.68	0.85	0.05	0.11	0.056	0.080
C. perfringens 28	0.84	1.23	0.17	0.44	0.010	0.011
C. perfringens 53	1.37	1.71	0.21	0.85	0.028	0.040
C. perfringens 54	1.19	2.05	0.26	0.38	0.032	0.032
C. perfringens 57	0.68	1.84	0.20	0.64	0.040	0.056
C. perfringens 532	1.23	1.64	0.15	0.54	0.053	0.064

[1]MIC is determined as the lowest concentration of tested compound that visibly inhibits bacterial growth.
[2]MBC is determined as the lowest concentration of tested compound that kills bacteria.

Table 2. Antibacterial activities (MIC and MBC) of α-bitter acids (a mixture of homologues), β-bitter acids (a mixture of homologues) and xanthohumol against anaerobic strains *Clostridium difficile* determined by dilution broth assay (MIC determination) followed by growth on solid media (MBC determination)

	Compound mg/mL					
	α-bitter acids		β-bitter acids		Xanthohumol	
	MIC[1]	MBC[2]	MIC[1]	MBC[2]	MIC[1]	MBC[2]
C. difficile 1810 (176)[3]	0.32	0.85	0.024	0.027	0.085	0.085
C. difficile 1811 (014)	0.77	1.37	0.080	0.171	0.085	0.085
C. difficile 1812 (001)	0.51	0.85	0.048	0.064	0.064	0.064
C. difficile 1813 (001)	0.51	0.85	0.021	0.021	0.064	0.064
C. difficile 1814 (078)	0.58	1.19	0.072	0.107	0.064	0.064
C. difficile 1815 (014)	0.77	0.85	0.021	0.016	0.064	0.064
C. difficile 1820 (001)	1.02	2.05	0.068	0.100	0.107	0.107
C. difficile 1821 (017)	0.51	1.02	0.032	0.032	0.032	0.064
C. difficile 1828 (176)	0.51	0.68	0.021	0.027	0.085	0.085
C. difficile 1829 (176)	0.34	0.68	0.027	0.053	0.053	0.064
C. difficile 1835 (176)	0.64	0.90	0.036	0.048	0.043	0.053
C. difficile 1836 (001)	1.02	1.02	0.096	0.212	0.075	0.085
C. difficile 1837 (005)	1.02	1.37	0.040	0.128	0.085	0.085
C. difficile 1838 (001)	0.43	0.68	0.080	0.208	0.032	0.040
C. difficile 2057 (176)	0.34	0.51	0.019	0.048	0.032	0.043
C. difficile 2058 (176)	0.68	1.02	0.027	0.064	0.032	0.048
C. difficile 2059 (176)	2.04	4.09	0.043	0.043	0.043	0.043
C. difficile 2062 (176)	0.51	1.02	0.027	0.048	0.032	0.064
C. difficile 2066 (176)	0.30	0.60	0.009	0.021	0.032	0.043
C. difficile 2083 (176)	0.68	1.02	0.024	0.044	0.064	0.064
C. difficile 2084 (176)	0.51	0.85	0.012	0.022	0.064	0.064
C. difficile 2085 (176)	1.02	1.70	0.053	0.128	0.064	0.064
C. difficile 2118 (176)	0.85	1.36	0.048	0.104	0.064	0.064
C. difficile 2132 (001)	1.02	1.36	0.024	0.044	0.043	0.053
C. difficile 2134 (001)	0.68	1.02	0.012	0.028	0.064	0.064
C. difficile 2137 (001)	1.02	1.36	0.048	0.136	0.064	0.064
C. difficile 2140 (001)	1.02	1.02	0.028	0.048	0.064	0.064
C. difficile 2142 (001)	1.02	1.02	0.048	0.104	0.053	0.064

[1]MIC is determined as the lowest concentration of tested compound that visibly inhibits bacterial growth.
[2]MBC is determined as the lowest concentration of tested compound that kills bacteria.
[3]Ribotype of *C. difficile* strain is in the brackets.

yet fully elucidated. Iso-α-acids pass the plasma membrane of cells by passive diffusion. Intracellular pH causes dissociation of iso-α-acids to anions and protons. Liberated protons lead to decreasing intracellular pH, disturbance of the transmembrane promotive force and to other metabolic effects (12). Because iso-α-acids are less effective than β-acids, they presumably act by different mechanisms (13). The principle of the antimicrobial effect of xanthohumol has likewise not been described. Mechanisms of resistance to bitter acids have also been described in some strains of *Lactobacillus brevis* and *Pediococcus*. They are based on a combination of mechanisms influencing the acidity inside the bacterial cell and are encoded by genes *horA* and *horC*.

A statistically significant factor affecting the MIC of α-bitter acids ($p = 0.01$) towards *C. difficile* was the tested strain, while other factors such as ribotype were not statistically significant ($p = 0.005$). Thirteen strains were found to be of ribotype 176, whereas 10 strains were of ribotype 001. These types are the most frequent ribotypes associated with CDI in Czech Republic. Ribotype 176 is related to ribotype 027 and can be misidentified as 027 by a commercial test (14). The antibiotic multiresistance of these ribotypes has been reported (15). In contrast to these facts, the susceptibility to bitter acids and xanthohumol is not ribotype-dependent.

Bitter acids and xanthohumol have very low water solubility. In animal model, the resorption of xanthohumol in the gut is only 11%, while 89% is staying in the gut and can affect gut microbial flora. Resorbed xanthohumol is metabolized in hepatic cells to glucuronides and other metabolites (16). Pharmacodynamic parameters of bitter acids are not known, but it can be expected that the toxicity of bitter acids and xanthohumol is very low. The subchronic oral administration of α-bitter acids to dogs was not associated with any signs of toxicity (17). Hops have been used as a component of beer for at least one thousand years. The concentrations of α- and β-bitter acids and xanthohumulone in beer are very low, because they are

chemically changed during the long boiling process. Although some people are drinking beer in large quantities, negative effects of beer except for those of alcohol were not recorded. All these parameters would increase the potential of these compounds for use as antimicrobials in the gut.

The antimicrobial activity of hop compounds was used for local therapy of oro-dental infections or acne vulgaris. Our results open the possibility of therapy of gut infections. Influencing gut microbial flora can have a positive effect in many syndromes, especially CDI. The current mainstays of therapy, metronidazole and oral vancomycin, have been used in CDI therapy for over 30 years. They are associated with clinical response rates of approximately 70–90% at the end of treatment. Metronidazole has been recommended for patients with mild-to-moderate CDI (18), whereas vancomycin is the recommended choice treatment for severe CDI.

The MIC values of xanthohumol and β-bitter acids for *C. difficile* determined in this study approach those found for metronidazole resistant *C. difficile* and are not excessively higher than the MIC values of metronidazole determined by the E-test for 10 non-toxigenic PCR ribotypes of *C. difficile* by Brazier et al. (19).

Despite a large attention being devoted to the therapy of CDI, the results are in many cases not satisfying, especially in recurrent infections. Hop components, especially xanthohumol, can pave a new way in CDI treatment because of their strong effect on *C. difficile* cells, very positive pharmacokinetics and low toxicity. One problem is their metabolic transformation to iso-α-bitter acids in case of α-bitter acids and isoxanthohumol in case of xanthohumol. Human gastrointestinal microbiota including anaerobic bacteria have been reported to transform the prenylflavonoid isoxanthohumol to 8-prenylnaringenin (8-PN) (20), antimicrobial effects of which are not known.

CONCLUSIONS

As part of the search for new agents with antibiotic properties, we tested the antibacterial properties of three types of purified hop compounds against three anaerobic pathogens, *Bacteroides fragilis, Clostridium perfringens* and *Clostridium perfringens*. The highest antimicrobial activity against all three microorganisms is exhibited by xanthohumol, followed by β-bitter acids, and the least effective against these anaerobic pathogens are α-bitter acids. Our data point out that hop extracts possess antibacterial characteristics and should be taken into consideration as potential antimicrobial agents with application in human and veterinary medicine. Plant antimicrobials, thus, represent a very interesting option for future use in therapy, but further research and development are needed to make them practically applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

8-PN: 8-prenylnaringenin; ATCC: American Type Culture Collection; BHI: brain–heart infusion; CLSI: Clinical and Laboratory Standards Institute; DMSO: dimethyl sulfoxide; EUCAST: European Committee on Antimicrobial Susceptibility Testing; FDA: Food and Drug Administration; MALDI-TOF: matrix-assisted laser desorption/ionization time of flight mass spectrometry; MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration; MRSA: methicillin-resistant *Staphylococcus aureus*; QIPD: Qualified Infectious Disease Product; VRE: vancomycin-resistant *Enterococcus*.

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