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Inhibitory Effects of Fresh Hops on *Helicobacter pylori* Strains

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Abstract

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The goal of this work was to provide evidence for the inhibitory effects of fresh hops on the growth of *Helicobacter pylori*. Fresh hops were homogenised after harvest and the homogenate was treated using pascalisation (treatment with high pressure). This homogenate was used for testing the growth inhibitory effects on strains of *H. pylori,* a pathogenic microorganism, isolated from patients suffering from gastritis or gastric ulcers. The tests demonstrated reliable inhibitory effects and open the possibility of hops being used as a supplement to the treatment of *H. pylori* infections.

Keywords: hops homogenate; *H. pylori*; antimicrobial effect

Helicobacter pylori is a spiral-shaped bacterium inhabiting the human stomach and was first described in 1906 by Krienitz (1906). Marshall and Warren (1984) suggested a link between *H. pylori* strains and gastritis and gastric ulcers. *H. pylori* is a spiral-shaped curved, gram negative, microaerophilic, flagellated bacterium inhabiting the surface of the mucous membrane that lines the inside of the human stomach. The bacterium produces large amounts of urease that eliminates the effect of the acid environment typical of stomach. This effect enables the bacterium to colonise the luminal surface. The main factors associated with its pathogenicity are considered to be urease, the production of cytotoxins, and the unique surface features of the bacterium. This bacterium causes gastritis, gastric ulcers, and is regarded as a potential cause of stomach carcinomas. The discovery of the causal relation between *H. pylori* and gastric illnesses led to a fundamental change in therapy. The current therapy is based on antibiotics combined with the medication affecting gastric juice secretion. Despite the therapy being very effective it is very hard to eradicate the pathogen in certain patients (Hentschel *et al.* 1993). The probable causes of this problem reside in that antibiotics have difficulties in penetrating the mucus on the surface of the mucous membrane, and in the increased resistance of *H. pylori* strains to antibiotics (Sedláčková 1996). Due to these problems, many researchers have studied alternative *H. pylori* treatment methods, e.g. herbs extracts applications. These methods were recently reviewed by Ayala *et al.* (2014). It is interesting that among the alternative treatments, hops or substances contained in hops were not mentioned.

Hops (*Humulus lupulus*) contains substances with various biological effects, e.g. resins, bitter acids, oils, and polyphenols, which are secondary metabolites generated in hop cones during ripening. Bitter acids are prenylated phloroglucinol derivatives. Alpha acids isomerise at higher temperatures in aqueous conditions into more soluble iso-alpha acids. Beta-acids do not contain the tertiary alcoholic group on

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the aromatic nucleus; therefore they are not able to isomerise. The presence of another isoprenyl side chain causes the molecule exhibiting as a whole a hydrophobic character. Therefore, β-acids are much less soluble in water compared to α-acids. Essential oils from hops have been examined in relation to their sedative effects for the treatment of sleep disorders (Dimpfel & Suter 2008). The most important groups of polyphenols are prenylated chalcones (prenylflavonoids), xanthohumol, and related compounds. Xanthohumol, in particular, has been examined in view of its potential anti-cancer effects (Biendl 2009). Dried hop cones contain, depending on the variety of hops, 2–17% α-acids and 2–10% β-acids. Among 11 Czech varieties, out of which cv. Vital (α-acids 12–16% w/w, β-acids 6–10% w/w) and cv. Agnus (α-acids 9–12% w/w, β-acids 4.0–6.5% w/w) have the highest contents of bitter acids. Traditional Saaz variety is specific due to a higher content of β-acids compared to α-acid (α-acids 2.5–4.5% w/w, β-acids 4.0–6.0% w/w) (Anonymous 2012). The total content of essential oils is 0.5–3%, of polyphenols 3–6%, and of xanthohumol 0.01–1% (Benitez *et al.* 1997).

The main goal of this work is to present the antimicrobial effects associated with a homogenate of fresh hops on the *H. pylori* strains.

MATERIAL AND METHODS

Hop samples. The hops homogenate was made from fresh green (not dried) hop cones followed by pascalisation; all procedures were carried out at the Food Research Institute Prague. Hops homogenates were prepared from the cultivars Agnus, Vital, and Saaz (Žatecký poloranný červeňák). The detailed description of the samples: there varieties of Czech hops, the homogenates prepared with or without the addition of ascorbic acid – natural pH or pH = 4.5. The hops were harvested in the years 2011 and 2012. The homogenates were pascalised at pressures 400 and 500 MPa for holding times 5 and 10 minutes. The numbers of samples: year 2012: 3 cultivars, 2 levels of pH, 2 methods of pascalisation: 3 × 2 × 2 = 12 samples; year 2011: 3 varieties, 2 levels of pH, 4 methods of pascalisation: 3 × 2 × 4 = 24, totally 36 samples (remark, 1 sample of cv. Vitá, year 2011) was damaged, totally tested 35 hops homogenates. Each sample was checked prior to testing for the presence of bacterial contamination by culturing in a liquid medium (Nutrient Broth No. 2; Oxoid, Basingstoke, UK) at 37°C for 24 h (0.5 g of sample in 10 ml of liquid medium). The hops samples were put into PE/ALUMINIUM pouches and vacuum packed. High pressure treatment (pascalisation) was made on a laboratory scale pressure unit (maximum pressure 550 MPa, holding time 30 min, chamber volume 2 l; producer ŽĎAS a.s., Žďár nad Sázavou, Czech Republic).

Strains of Helicobacter pylori. *H. pylori* strains were obtained from clinical specimens taken from patients at the University Hospital Motol, Prague, Czech Republic (27 strains) and Thomayer Hospital, Prague, Czech Republic (5 strains) and from the DSM collection of organisms (DSM No. 21031). Thirty three strains of *H. pylori* were used.

The strains from patients were identified using a Bruker MALDI TOF mass spectrometer (Bruker UK Ltd, Coventry, UK). The cultivation was performed on Columbia blood agar (BioRad, Marnes-la-Coquette, France) supplemented with 10% horse blood, in a microaerophilic atmosphere with 10% CO_2 at 37°C for 72 hours. The strains were stored in a system ITEST KRYOBANKA K for bacteria (ITEST PLUS, Hradec Králové, Czech Republic) at –70°C. Resuscitation was performed on Columbia blood agar.

Determination of MIC of hop homogenates. The determination of minimum inhibitory concentration (MIC) was carried out using the agar dilution method. The autoclaved Columbia agar base was cooled to 38–40°C and then supplemented with the horse blood and the hops homogenate until the desired concentration of hops in the culture medium was obtained. We added 0.1–3.2 g/l (in doubled dilution 0.1, 0.2, 0.4, 0.8, 1.6, 3.2).

While stirring, the prepared culture medium was poured onto 90 mm diameter (19 ml/dish) Petri dishes. When the temperature further decreased, the prepared plates were inoculated with freshly revived *H. pylori* strains. Each strain was, apart from on agar plates with different concentrations of hops homogenate, inoculated on Columbia blood agar plate for cultivation without hops homogenate as a growth control.

The inoculated plates were then cultured in microaerophilic atmosphere with 10% CO_2 at 37°C for 72 hours. The MIC was determined as the lowest concentration of hops homogenate on the plate with no growth of the tested strain. The results were evaluated only for strains with positive growth on parallel run control plates.

Bitter acid of hops MIC prediction. The same method as that used for the determination of the

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MIC of the hops samples was also used for hops bitter acids. Instead of the hops homogenate, a solution of bitter acid dissolved in alcohol, in the quantity and concentration to obtain the desired concentration of bitter acids in the culture medium, was added to the autoclaved Columbia agar base and cooled to 38–40°C. We added β-acids (0.75–6 mg/l) and α-acids (1.5–24 mg/l) (also doubled dilution). Other procedures were the same. The samples of α- and β-bitter acids were obtained from a commercial preparation of hops extract (CO_2 hop extract; Hopsteiner, Nuremburg. Germany) in accordance with the procedure described by Krofta *et al.* (2012).

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-acid (purity 99.7%) isolated through crystallisation from the solvent mixture. The alpha fraction was used for the preparation of α-acids with a purity of 93% (the remainder consisting of non-specific resins).

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

RESULTS

MICs were determined for a total of 35 samples of the hops homogenate. The average MIC for 964 measured samples of hops homogenate was 0.47 g/l with limit values of 0.15 and 1.03 g/l. Statistically significant factors were evaluated in relation to the harvest year ($P = 8.74 \times 10^{-35}$) and strain *H. pylori* ($P = 3.53 \times 10^{-18}$). In contrast, the differences between the hops varieties were not statistically significant.

The average MIC of alpha bitter acid measured in all strains of *H. pylori* was 8.17 mg/l (limit values of 3.0 and 24.0 mg/l); for beta bitter acid the average MIC was 3.05 mg/l (limit values 1.5 up to 6.0 mg/l). The results are graphically presented in Figures 1 and 2. The content of alpha bitter acids ranged from 0.46 to 3.27 mg/l that of beta bitter acids 0.63–2.20 mg/l, depending on the variety and harvest year. The contents of bitter acids, total polyphenols and xanthohumol in the homogenates are given in Table 1. The concentration of bitter acids in the culture medium at an average MIC, depending on the variety of hops and harvest year, is given in Table 2.

DISCUSSION

It is often difficult to obtain the cultures of *H. pylori* from patients. The diagnosis and treatment are based on indirect evidence, such as the determination of *H. pylori* antigen in stools.

Gastric mucosal biopsy is performed only if the infection does not respond to therapy, at which time it becomes necessary to determine the antibiotic sensitivity. Therefore, the tested strains represent

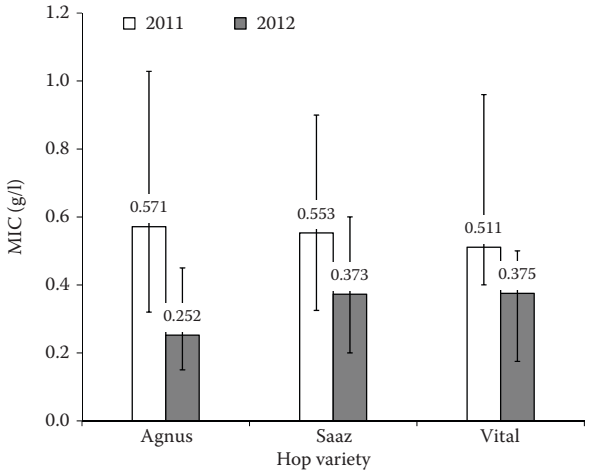

Figure 1. Average value of MIC for hops varieties Agnus, Saaz (Zatecky poloranny cervenak) and Vital, harvest years 2011 and 2012 (columns are averages, bars represent the minimum and maximum value)

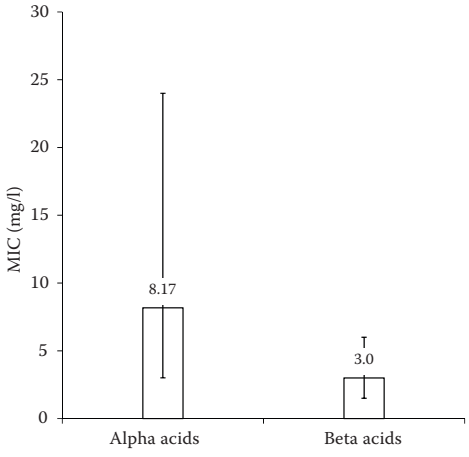

Figure 2. Average MIC alpha and beta bitter acids measured in all strains of *H. pylori* (columns are averages, bars represent the minimum and maximum value)

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Table 1. Content of bitter acids and polyphenols in hops homogenates (mean values and standard deviations)

Substance	Harvest 2011			Harvest 2012		
	Saaz	Agnus	Vital	Saaz	Agnus	Vital
α-Acids (g/100 g)	0.46 ± 0.02	3.07 ± 0.16	3.10 ± 0.17	0.67 ± 0.03	2.95 ± 0.13	3.27 ± 0.01
β-Acids (g/100 g)	0.63 ± 0.03	2.13 ± 0.11	2.20 ± 0.12	1.13 ± 0.05	1.70 ± 0.10	1.98 ± 0.11
Total polyphenols (mg/g)	5.44 ± 0.82	9.99 ± 1.39	9.46 ± 1.49	7.80 ± 1.12	6.32 ± 0.87	6.62 ± 0.90
Xanthohumol (mg/g)	0.32 ± 0.02	0.88 ± 0.05	0.92 ± 0.04	0.47 ± 0.03	1.58 ± 0.09	1.38 ± 0.08

Table 2. Concentration of bitter acids in the culture medium at an average minimum inhibitory concentration (MIC), depending on the variety of hops and harvest year (mean values and standard deviations)

Parameters	Harvest 2011			Harvest 2012		
	Saaz	Agnus	Vital	Saaz	Agnus	Vital
Average MIC (g/l)	0.55 ± 0.14	0.57 ± 0.12	0.51 ± 0.12	0.37 ± 0.05	0.25 ± 0.05	0.38 ± 0.02
α-Acids concentration at MIC value (mg/l)	2.55 ± 0.64	17.5 ± 3.54	15.85 ± 3.64	2.5 ± 0.32	7.44 ± 1.54	12.25 ± 0.65
β-Acids concentration at MIC value (ng/l)	3.49 ± 0.88	12.15 ± 2.46	11.26 ± 2.58	4.23 ± 0.55	4.27 ± 0.89	7.42 ± 0.40

a population of *H. pylori* that were likely possess a greater resistance to therapy.

The determination of MIC of *H. pylori* bacterial strains is accompanied by a series of technical problems. The standard dilution method cannot be used because *H. pylori* does not grow in liquid broth.

The disk diffusion method is also problematic because bitter acids are not soluble in water, therefore a very small amount diffuses into the culture medium. The resulting inhibition zones are small and difficult to interpret. The studies testing the effects of bitter acids using this method were only able to show the presence of bacterial growth inhibition (Haas & Barsoumian 1994; Oshugi *et al.* 1997; Bhattacharya *et al.* 2003).

In the present study, we developed a dish dilution method that eliminates the difficult problem of diffusion of a homogeneous dispersion of the hops homogenate throughout the entire volume of the culture medium. To prevent chemical changes of bitter acids at high temperatures, the hops homogenate and hops bitter acids were added at the lowest temperature possible, together with horse blood.

The test file strains represented a population of pathogenic microorganisms obtained in less than two years. The microorganisms differed in the growth morphology, some had to be revitalised. Nonetheless, the measured values of MIC still ranged within one order of magnitude.

MIC differences between *H. pylori* strains were statistically significant but their variability was significantly lower compared to the susceptibility of other bacterial pathogens to antibiotics. One item of explanation might be other sort of inhibitory effects of bitter acids, completely different from the effects of antibiotics (Matoulková *et al.* 2010).

A number of studies have described the inhibitory effects of the compounds isolated from hops on various microorganisms, the α- and β-acids receiving the most attention (Schmalreck *et al.* 1975). Antimicrobial activity has been demonstrated, in particular, on Gram-positive cocci (Haas & Barsoumian 1994), hereinafter 'mycobacteria' (Chin *et al.* 1949), and parasites (Nutter *et al.* 1998). With the exception of *H. pylori* (Oshugi *et al.* 1997), α- and β-acids are not effective on Gram-negative bacteria (Bhattacharya *et al.* 2003) or fungi (Sale *et al.* 1949). The activity of bitter acids on several fungi (Mizobuchi & Sato 1984) and protozoa (Srinivasan *et al.* 2004) has been described. The main antimicrobial activities have been ascribed to β-acids (Gouin 1958). Beta acids alone can inhibit the growth of *Listeria monocytogenes* strains in combination with antimicrobial agents used in the food industry, which further increases their effects (Shen & Sofos 2008). The mechanism of bitter acids action has not yet been fully elucidated. It is known that iso-α-acids and the products derived from α-acids penetrate the cell wall and plasma membrane and cause intracellular acidification. This leads to a reduction in the activity of certain enzymes and stops the intake of nutrients. The resistance of certain strains of *Lactobacillus brevis* to hops bitter acid is probably caused by a combination of mechanisms influencing the acidity inside the bacterial cell (Matoulková *et al.* 2010).

In order to identify other compounds with a higher efficiency than β-acids, substances with greater water solubility (e.g. iso-α-acids, xanthohumol) have been tested. Iso-α-acids were compared with β-acids but were found to be less effective (Srinivasan *et al.* 2004). Xanthohumol probably has a broad spectrum

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of anti-infective properties. It can act on Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus mutans*), viruses (cytomegalovirus, herpes simplex virus type 1 and type 2, and human immunodeficiency virus 1), the fungus (*Trichophyton* spp.), and the malaria protozoa (*Plasmodium falciparum*). The mechanisms of inhibition were the subject of research by Gerhäuser (2005). Most of the works used the disk diffusion method to determine the antimicrobial activity or measured the changes in optical density. Shen and Sofos (2008), when testing antimicrobial activity of bitter acids, determined the minimum inhibitory concentration (MIC) and evaluated the growth parameters using the plate dilution method.

All these works focused on the antimicrobial properties of substances contained in hops for increasing the shelf life of foods. Hops were tested as a treatment for acne using the plate dilution method and MIC on the strains of *Propionibacterium acnes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Kocuria rhizophila*, and *Streptococcus pyogenes* (Yamaguchi *et al.* 2009). The results showed that the inhibitory effects of β-acids were comparable to the effects of antibiotics.

The observed higher efficiency of beta bitter acids is consistent with the data presented in the literature. The average MIC values of β-acids were, on average, lower by a factor of three in comparison with those of α-acids. Beta acids have greater inhibitory effects on the *H. pylori* strains than α-acids. This result is consistent with the literature regarding the effectiveness of β- and α-acids on fungi and protozoa (Srinivasan *et al.* 2004).

As the most important structural feature of β-acids in terms of antibacterial properties is considered the presence of three isopropenyl nonpolar chains that make the entire molecule highly hydrophobic; the antibacterial activity is greater in acidic environments that suppress the dissociation of β-acids (Schmalreck *et al.* 1975; Simpson & Smith 1992).

The MIC values obtained represent the cumulative effect of the substances contained in fresh crushed hops capable of affecting nearby *H. pylori* cells. The inhibitory effects are very strong and the values measured approximate the effects of antibiotics.

The contents and compositions of secondary metabolites in hops and thus the levels of potential inhibitory substances are primarily dependent on the variety of hops, the soil, and climatic growing conditions. It is therefore understandable that the inhibitory effects of the individual crop varieties can be different.

Statistically, the influence of the harvest year has been shown. The differences between the measured values of MIC are very small and are in the range of one order of magnitude. The bitter cultivars Agnus and Vital are, from the point of view of α- and β-acids and other substances, comparable. Significantly lower amounts of α-and β-acids are associated with the aromatic cv. Saaz (Žatecký poloranný červeňák) (Anonymous 2012). However, the measured values of MIC do not show any statistically significant difference between the cultivars. Also, the differences in the average MIC values between the 2011 and 2012 harvest do not correspond to those between the contents of bitter acids in the hops coming from these harvest years (Table 1 and Figure 1).

The predicted higher antimicrobial activity of beta bitter acids compared to that of alpha bitter acids corresponds with the literature data (Srinivasan *et al.* 2004). Provided the bitter acids represent the main effective substances (Bhattacharya *et al.* 2003), there should be substantially greater differences in MIC for cv. Saaz hops samples that contain substantially lower bitter acids content. This fact can be explained by synergistic effect of alpha and beta bitter acids. Synergistic effect is applied as a standard in the therapy of infectious diseases combining several antibiotics. The substance of this method is the simultaneous effect on different parts of the bacterial cell structure. Combined antibiotics differ by the place of the effect on the cell body and by the chemical composition of the effective molecules. In the case of bitter acids, their chemical structure is very similar. The mechanism of the effect is also very similar. Therefore, their synergistic effect is not very probable. Hop contains also prenylflavonoids with the main component xanthohumol and several other components. Based on our results, it is very probable that there are other not well defined components possessing antibacterial effects.

Our results create the starting basis for further hops research as a potential source of components having an anti-infective effect. Current antibiotic crisis gives the opportunity for intensive search for antimicrobial components and therapies of infections. Hop as a whole can be regarded as such a source.

CONCLUSIONS

This study demonstrated the inhibitory effects of hops on *H. pylori* strains isolated from patients with gastritis caused by *H. pylori*. The results obtained are important and strongly suggest that hops can be used

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as an adjunct or even an alternative to antibiotics in the treatment of *H. pylori* infections.

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