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A novel approach for identification of biologically active phenolic compounds in complex matrices using hybrid quadrupole-orbitrap mass spectrometer: A promising tool for testing antimicrobial activity of hops

Martin Dušek [a,*], Vladimíra Jandovská [a,c], Pavel Čermák [b], Alexandr Mikyška [a], Jana Olšovská [a]

[a] Research Institute for Brewing and Malting, Prague PLC, Lípová 15, CZ-120 44 Prague 2, Czech Republic
[b] Thomayer's Hospital, Vídeňská 800, CZ-140 59 Prague 4, Czech Republic
[c] Faculty of Science, Charles University in Prague, Albertov 6, CZ-128 43 Prague 2, Czech Republic

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ABSTRACT

The phenolic compounds, secondary metabolites of hops represent a large family of compounds that could be subsequently divided into smaller groups based on the similarities between their chemical structures. The antibacterial, antifungal and antiviral properties of hops are well known, but there is a lack of information about antimicrobial activities of individual hop compounds.

This study was carried out with an objective to identify compounds present in hops that have potential antibacterial activity. In the first stage of experiment, the active compounds with potential antimicrobial activity had to be extracted from hop cones. Therefore, minced hop cones were applied on solid growth medium inoculated with *Staphylococcus aureus*. The active substances that migrated into the medium created an inhibition zone. In the second stage of experiment, the inhibition zones were cut out from Petri dishes, active compounds were extracted from these zones and consequently analyzed using LC-HRMS. These complex assays were developed and optimized. The data were acquired by using a quadrupole-orbitrap hybrid mass spectrometer by targeted-MS2 experiment in both ionization modes. The MS method has been developed as a screening method with a subsequent fragmentation of compound of interest on the base of inclusion mass list. The unknown compounds extracted from inhibition zones have been identified either by searching against a database or their structure has been elucidated on the basis of their fragmentation spectra. On the basis of this experiment the list of active compounds with potential anti-microbial activities was enhanced.

1. Introduction

Humulus lupulus belongs to the family Cannabaceae, is a flowering plant whose female gender produces the cones also called seed cones or strobiles. Non-fertilized strobiles are primarily used a flavoring and antibacterial agents in brewing. Moreover, apart from the known role in brewing beer, hops are also used in herbal remedies as a treatment to cure diseases or ailments like anxiety, inability to sleep (insomnia and other sleep disorders), restlessness, tension, excitability, nervousness, and irritability [1]. Olšovská et al. [2] summarized the known biological activity of all three basic groups of hops secondary metabolites (resins, essential oils and polyphenols), however, there is a lack of information about antimicrobial activities of individual hop compounds.

1.1. Morphology and chemical composition of hop cone

The flowers of female hop plants form the hop cones that appear as side arms that develop along the stem. These hop cones are made of strings, bracts, bracteoles and lupulin glands. The stipular bracts and seed-bearing bracteoles are leaf-like structures that are attached to the central axis or string surrounding the entire cone. The bracts are pointed at the tip, the bracteoles are rounded. The bracts perform as protective organs and contain few lupulin glands whereas the bracteoles are winged organs containing a wealth of lupulin glands, particularly at the bottom in a pocket-like fold. The lupulin glands form tiny yellow sacs found at the base of the petals of the hop cone.

* Corresponding author.
E-mail address: dusek@beerresearch.cz (M. Dušek).

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In hops, during their maturation lupulin glands produce characteristic hop secondary metabolites (resins, essential oils and prenylflavanoids), which are placed on the bracteoles of the cone. The hard hop resins are mainly composed of oxidation products of the soft resins which are produced during the maturation, post-harvest processing and storage of hop cones. The natural hard resins contain xanthohumol which is the most abundant prenylated chalcone present in hops. The soft resins include alpha-bitter-acids (humulones), which are a mixture of humulone, colupulone and adhumulone, beta-bitter acids (lupulones) including lupulone, colupulone and adlupulone, and uncharacterized soft resins.

The polyphenols are mainly concentrated in the green parts of the hop cone (string, bracts, bracteoles) but the distribution of the individual groups of the polyphenols in each of these parts varies as well. Polyphenols are hop secondary metabolites containing more than one phenolic hydroxyl group. Polyphenols involve these groups: flavonols, flavan-3-ols, phenolic carboxylic acids and others as prenylflavonoids, which are the most important group of polyphenols [3,4].

1.2. The principles of testing antimicrobial activity of plant matrices

Several tests can be used to evaluate antibacterial activity. Tests are divided into two groups: qualitative diffusion and quantitative dilution methods. Qualitative testing of antimicrobial properties use diffusion method and agar diffusion method and the quantitative methods include broth dilution method, agar dilution method and microdilution method.

These methods measure the minimum inhibitory concentration (MIC), the lowest concentration that inhibited the growth of bacteria, or minimum bactericidal concentration (MBC) the lowest concentration of the active substance that produces total death of bacteria [5,6].

When using broth dilution method, an active agent is added to the tube with broth and serial dilutions are made in a required concentration range. Subsequently, a suspension of tested microorganism is added to each tube [7]. The principle of microdilution method is the same as broth dilution method; testing is carried out on many small trays with suitable concentration range of active agents and serial dilutions.

The diffusion methods validate bacterial sensitivity to antimicrobial agents. Disc diffusion method is based on placement of filter paper disc containing active agents on the inoculated surface of Petri dishes. Antimicrobial agents create concentration gradient around the disc and it shows as an inhibition zone after incubation. Antibacterial activity is evaluated by measuring the radius of the inhibition zones or taking photos of Petri dishes and processing them using software [8,9]. A simpler variant of the disc method is agar diffusion method where a sample is applied to wells which are made in an agar plate. After inoculation and incubation an inhibition zone is evaluated due to diffusion of the active agent into the agar. The zone can be measured as in the previous method. Dorman and Deans [10] applied agar diffusion method for testing antimicrobial activity of volatile oils extracted from herbs and plants including compounds such as thymol, eugenol (these compounds are also present in hops) against 25 bacteria.

1.3. Advantage of mass spectrometry in hop analysis

The hop is a very complex matrix. The most important groups of substances are essential oils, soft resins (α-bitter acids), beta acids, hard resins (xanthohumol) and a wide group of polyphenols. These compounds, except for essential oils, are analyzed using liquid chromatography, nowadays usually coupled to mass spectrometry (LC-MS) [11]; nevertheless each compound requires a special method of preparation. This preparation usually employs extraction of sample using a polar solvent, e.g. ethanol, methanol, acetone followed by sample clean-up with solid phase extraction (SPE), which is optimized for the chemical properties of a relatively tight group of analytes. Callemien et al. [12] extracted the polyphenol resveratrol from hops by employing solid/liquid extraction using ethanol: water after removal of hydrophobic bitter compounds by toluene and cyclohexane. Measurement was carried out by HPLC-MS/MS. For determination of metabolite profile in common cultivars of hop, Farag et al. [13] used LC-MS. They extracted hop resins from milled hop cones, purified on SPC 18 cartridge and analyzed them by ESI-FTICR-MS. Zhang et al. [14] used for direct characterization of bitter acids liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. The hop underwent ultrasonic extraction with ethanol and this extract was concentrated. A more universal approach is based on extraction procedure using QuEChERS principles [15] and is usually used for sample preparation prior to LC-MS analysis. Dušek et al. [16] used this method to isolate beta-bitter acids and their oxidation products (humulones) in beer prior to analysis by LC-MS/MS.

The aim of this work was isolate and identify compounds with potential antimicrobial activity. Sensitive and unequivocal methodology for determination of these active compounds in this complex matrix has been developed using the high-resolution mass spectrometer allowing for accurate mass measurements.

2. Materials and methods

2.1. Chemicals and material

The standards of xanthohumol (XN), isoxanthohumol (IXN), 8- and 6-prenylnaringenin (8-PN and 6-PN), catechin (C), epicatechin (EC), rutin (RUT), quercetin (Q) and internal standard probenecid (ISTD) were purchased from Sigma Aldrich (St. Louis, MO, USA) and individually dissolved in acetonitrile to obtain stock solution of 1 mg/mL. The mixture of purified beta-bitter acids (lupulone, colupulone and adlupulone) was obtained from the Hop Research Institute Co., Saaz, Czech Republic.

Acetone (anal. grade, $\geq$99.98%) and sodium chloride (anal. grade) were obtained from Lach-ner (Neratovice, Czech Republic). Acetonitrile (LC-MS grade, $\geq$99.9%) and formic acid (LC-MS grade, 98%) were obtained from Sigma Aldrich (Steinheim, Germany). Magnesium sulfate (anal. grade, $>$98%) was obtained from Penta (Prague, Czech Republic). Pure water was obtained from a Milli-Q purification system (Merck-Millipore).

2.2. Preselection of biological active compounds on Petri dishes with hops spot

A one centimeter (approx. 0.4 in.) wide hole that was cut out from the layer of Mueller-Hinton agar (Bio-Rad, France) in a Petri dish (diameter 90 mm, MEDLAB, Czech Republic). The hole was filled with minced hop cones and the minced hop was subsequently fixed in position by molten agar. The whole surface was inoculated with 0.5 McFarland suspension of *Staphylococcus aureus* CCM 3953. The dish was cultivated at 37 °C for 24 h. The zone of inhibition was cut out, sliced, frozen and lyophilized.

2.3. Extraction and sample preparation

Extraction of potential biologically active compounds from lyophilized solid growth medium was carried out by an acetone-water mixture (70/30, v/v) and re-extracted with acetonitrile, and