DE GRUYTER OPEN
Open Chem., 2015; 13: 1157–1171

Research Article

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Supercritical carbon dioxide hops extracts with antimicrobial properties

DOI: 10.1515/chem-2015-0131
received February 23, 2015; accepted July 27, 2015.

Abstract: Extracts obtained from hops (*Humulus lupulus* L., Cannabaceae) by supercritical fluid extraction (SFE), SFE followed by isomerization, as well as by conventional technique, were investigated for their chemical composition and antibacterial activity against selected foodborne pathogens and microorganisms capable to cause the food spoilage. The antibacterial activity of the extracts was compared with the antibacterial activity of xanthohumol, compound known for its broad pharmacological properties, isolated from the raw material remained after the SFE. Xanthohumol (XH, 96%) proved to posses the most prominent activity against all the tested strains, with the MIC values ranged between 2.5 and 20 μg mL^{-1}. Supercritical hops extract and potassium isomerized supercritical hops extract showed strong antibacterial activity against the tested strains as well. *Escherichia coli* was not affected by the extracts, meaning that their oral admission would not cause the same problem as antibiotic application in intestinal flora. The chemical composition of the investigated hops extracts was analysed by GC-MS. Contents of α-acids, β-acids, iso-α-acids and xanthohumol in the samples were determined by HPLC.

Keywords: supercritical hops extract, isomerized hops extract, supercritical CO_2 extraction, xanthohumol, antibacterial activity

1 Introduction

Since ancient times, hops (*Humulus lupulus* L. Cannabaceae) have been used in traditional medicine for their anti-inflammatory, antiseptic, antidiuretic, aphrodisiac, hypnotic, sedative, and stomachic properties. The German Commission E approved a monograph on hops for use in mood and sleep disturbances. Similar indications are described in an ESCOP (European Scientific Cooperative on Phytotherapy) monograph. Today, a wide range of the preparations containing hops extracts or hops-derived products are available on the market, in particular for use in the phytotherapy of sleep disorders or pain relief and in postmenopausal therapy. Increasing evidence reveals that the so-called hops bitter acids, which represent up to 30% of the total lupulin content of hops, prenylflavonoids and xanthohumol exhibit interesting effects on human health [1-4]. Also, it is well-known that hops and the brewing industry are strongly linked. Compounds derived from hops have several effects on beer. The resinous compounds, α- and β-acids give a bitter taste to beer, while essential oils are responsible for the specific aroma. Tannins may play a part in the clarification of beer by precipitating proteins during boiling. Hops bitter acids may protect the beer against lactic acid bacteria (genera of *Lactobacillus* and *Pediococcus*) and add to the improvement of the stability of beer foam [5]. Much of attention has been devoted to the polyphenolic content of hops, and specific compounds, such as xanthohumol and 8-prenylnaringenin, have been identified as multipotent bioactive compounds. According to the pronounced bioactive effects associated with most of the hops secondary metabolites, the pharmaceutical industry has considered it as a potential source of new plant derived medicines.

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Hops use as beer additive goes back to the German monks in the 12th century. It was quickly realized that hops not only added bitterness and aroma to beer, but also played an important role as a preservative. In the early 1900s, Brown and Clubb first described the antiseptic properties of hops [6]. Subsequently, α- and β-acids (humulones and lupulones), constituents of the essential bitter resin of hops, were identified as strong antibiotics against Gram-positive bacteria.

In agreement with the current trends, the beginning of the 21st century has been marked by a greater demand of by the population for natural products. Therefore the use of natural compounds, particularly in providing the food safety and in prevention of food spoilage, becomes very frequent [7]. Currently some natural antimicrobials, including plant extracts to control foodborne pathogens, have been approved by The U.S. Deptartment of Agriculture Food Safety and Inspection Service (USDA-FSIS) as a possible method for reducing pathogenic bacteria in meat and poultry products (USDA-FSIS 2008) [8]. It is well-accepted fact that nutrition plays a prominent role both as a risk factor but also as a measure to prevent various kinds of diseases, such as cancer, and coronary heart diseases. Recent estimates from the Centres for Disease Control and Prevention in the United States suggest that there are 76 million cases of food-borne illness in the US each year, which result in about 5000 deaths. The foodborne illnesses are associated with *Bacillus cereus*, *Listeria monocytogenes* (capable of multiplication even at the temperature of freezing), *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Toxoplasma gondii*. Hence our interest is to explore whether the hops obtained by supercritical carbon dioxide extraction, rich in bitter compounds, could be used as antimicrobial agents against some of the most common foodborne pathogens and also against microorganisms capable of causing the food spoilage, namely against *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, and *Lactobacillus sp.*

L. monocytogenes is a well-known zoonotic causative agent of listeriosis and is an extremely dangerous disease for humans and animals and manifests as encephalitis, miscarriages or septicemias. It is present in vegetables (onion, spinach, green salad, melon, *etc.*) and also in meet, milk and cheese. *L. monocytogenes* can survive and grow under adverse conditions including refrigeration temperatures, low pH, and high salt concentrations. Although listeriosis outbreaks are not common, its fatality rate is high (20% to 40%) for high-risk groups such as pregnant women, neonates, and immunocompromised adults (ILSI Research Foundation/ Risk Science Inst. 2005). Because of its ability to withstand sanitizing and food processing procedures, *L. monocytogenesis* has found a place in the food industry as an indicator organism in evaluating the efficacy of sanitation procedures and good manufacturing practice. Therefore, *L. monocytogenes* continues to be a major concern to the food industry and public health, and the investigation of new antilisterial agents is needed. *B. cereus* is widely present in the environment and besides food spoilage it is capable of causing mild diarrhea. *S. aureus* produces enterotoxin which causes intoxication. Lactobacilli are "good" bacteria responsible for aroma and consistency of cheeses and other milk products. But, it is known that these bacteria represent the main beer contaminant and might affect yeast performance, causing the losses in ethanol yield, and form undesirable off-flavors. *E. coli* is the only gram-negative species used in this investigation and it is a causative agent of different kinds of infections including foodborne diseases.

The hops compounds are weak acids classified into α-acids and β-acids. α-Acids are represented by humulone and its congeners (cohumulone, adhumulone, prehumulone and posthumulone). The β-acids are lupulone and its congeners (colupulone, adlupulone, prelupulone and postlupulone). The α-acids and β-acids have alicyclic structures (2,4-cyclohexadiene-l-one); their congeners differ by the nature of the acyl side chain. α-Acids were found to be present in beer at concentration levels of 150–200 μg L^{-1}, while β-acids were absent. Many content studies have been performed on the chemical composition in differently prepared hops extracts, and extracts that undergo the chemical changes, resulting in the isomerization of α-acids, humulones into corresponding isomers. The presence of the isomerized α-acids is of the great importance for brewing industry because of the contribution they make to the desired bitterness of beer. Besides the influence on the olfactory properties of beer, hops bitter acids have interesting and various pharmacological properties. For example, the antioxidative activity of hops bitter acids and their analogues may contribute to a cancer preventive effect since they can quench free radicals which cause oxidation of the DNA in the body and thus cause genetic defects. Humulon has been known for its preventive effect against osteoporosis and for capability to hinder the growth of certain leukemia cells especially with the combination of vitamin D. The results of one recent study confirmed that beer components are protective against the genotoxic effects of heterocyclic amines on target organs associated with tumorigenesis "in vivo" [1,9]. It is also known that the isomerized bitter acids (isohumulones) might prevent

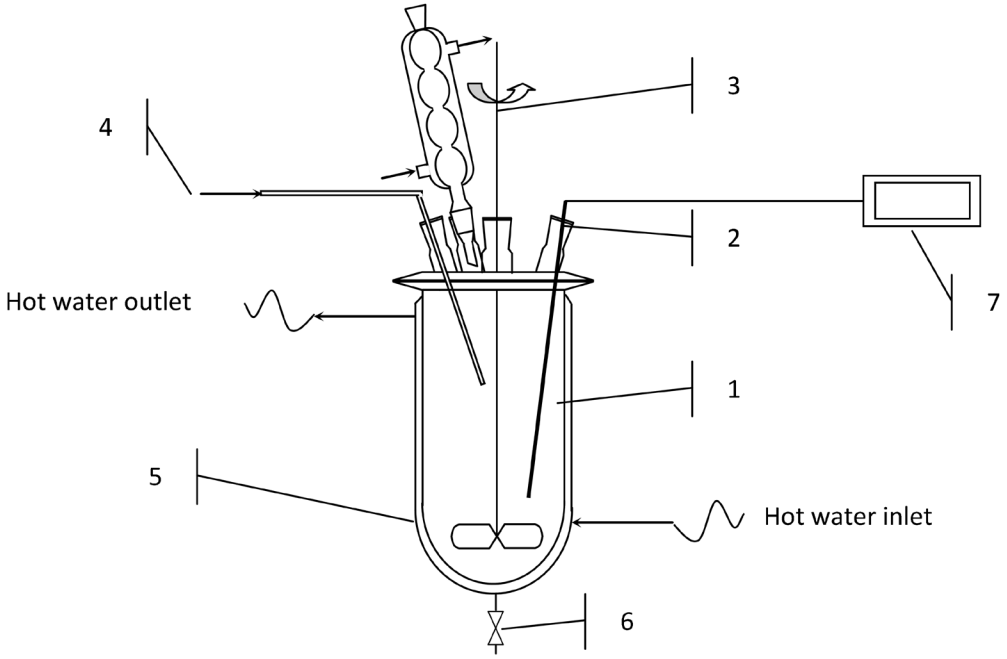

Figure 1: A schematic diagram of experimental apparatus: 1 – glass reactor V – 1,5 dm^3, 2 – thermocouple, 3 – mechanical stirrer, 4 – inert gas inlet, 5 – water heating jacket, 6 – drain valve, 7 – temperature digital display).

the developments of noninsulin dependent diabetes and hyperlipidemia, improving insulin sensitivity in patients with type II diabetes [2]. Hops extracts have long been known to have antimicrobial properties. The preservative properties of hops have been investigated for many years, and despite some reports on the antibacterial activity of hops oil, the bitter acids seem to be the main active compounds. As stated in the literature [10], the hops bitter acids inhibit mainly Gram-positive bacteria, including some species of *Bacillus, Micrococcus, Staphylococcus, Mycobacterium* and *Streptomycetes*, whereas yeast (*Saccharomyces cerevisiae*) and *Escherichia coli* are not affected. The mechanism of the lipophilic region of the cell membrane represents the target site for the antibacterial action of hops bitter resins. Consequently, the antibiotic properties were shown to depend mainly on the hydrophobic parts of the molecules and increased with decreasing solubility [11,12]. This hypothesis was confirmed in *B. subtilis*, in which lupulone, humulone, and isohumulone caused cell wall lesions by incorporating into the cytoplasmic membrane. This activity resulted the inhibition of active transport of sugars and amino acids and, subsequently, led to inhibition of cellular respiration and synthesis of proteins, RNA, and DNA. Apparently, it is the same mechanism by which *trans*-isohumulone inhibits the growth of the beer-spoilage bacterium *Lactobacillus sp*. The iso-R-acids act as a mobile carrier for ionophores, catalyzing electroneutral influx of the undissociated molecules, as well as their internal dissociation and efflux of their complexes with divalent cations such as Mn^{2+}. The resulting loss of the proton gradient inhibits the uptake of sugars and causes starvation in the bacterial cells. Since hops acids are weak acids and only the undissociated forms are active, the antibacterial properties fall with higher pH values. Furthermore, the potency is enhanced by increasing the hydrophobicity of the molecules, as determined by the acyl side chain length and the number of prenyl groups [13]**.** Lupulones, lupulone, colupulone and adlupulone, have been reported to have greater antimicrobial activity than the iso-α-resines (humulones). Namely, the water-insoluble β-acid lupulone was about twice as active as the α-acid humulone, whereas the soluble iso-α acid isohumulone was less active than lupulone [14]. Hops acids may behave as either bacteriostatic substances or bactericides, depending on the conditions employed.

The aim of this study was to investigate whether the different hops extracts, supercritical hops extracts, extracts obtained by isomerization of supercritical hops extract, and hops extract obtained by procedure of the classical extraction, for comparison purpose, could be used as antimicrobial agents against the before mentioned foodborne pathogens and also against microorganisms capable of causing food spoilage.

In addition, under the conditions used in extraction with supercritical carbon dioxide for preparations the hops extract for brewing industry, a large group of the biologically active prenylflavonoids (prenylchalcones and prenylflavanones) remain in the spent material. The most

interesting compound in the material left behind after the hops extraction for breweries, due to its limited degree solubility in non-polar carbon dioxide of used supercritical parameters, is xanthohumol (XN) with the unique and the significant pharmacological properties. Taking into account that hops is the only known natural source of this substance, and that XN shows strong anticancer effect, possesses antioxidizing properties, and exerts the confirmed (by *in vitro* tests) antifungal, antibacterial and antiviral activities including that against HIV-1 [15,16], the aim of this study was to investigate antimicrobial properties of XN and extract enriched XN content obtained in a secondary extraction of the residue after hops extraction with supercritical carbon dioxide [17]. Furthermore, by performing the detailed investigation regarding the chemical composition of the different hops extracts, the aim was to give insight into which components might be considered responsible for such an activity.

2 Experimental procedure

2.1 Plant material

Hops cones, Marynka variety, were harvested in Lubelski region (Poland) in autumn 2010. Fresh cones were dried and commercially pelletized. Such pellets were used for hops extract production process. The commercial hops pellets, type 90, (Marynka variety) were produced by New Chemical Syntheses Institute (INS), in Puławy. Contents of α-acids and dry matter were 7.4% wt and 7.9% wt, respectively.

2.2 Preparation of supercritical carbon dioxide hops extract

Hops extract, HE, was produced from hops pellets by SFE using carbon dioxide at pressures up to 30 MPa and at temperatures up to 50°C at the New Chemical Syntheses Institute, in Puławy, Poland. The extraction process was carried out in the INS commercial plant. This plant consists of four extraction vessels, each with a capacity of approx. 2.6 m^3. There are two separation stages at a pressure of approx. 6 MPa for extract and water separation. The plant can operate within a pressure range of 15–30 MPa and at temperatures up to 100°C. Extraction products were analyzed by GC, HPLC and Ultra High Performance Liquid Chromatography (U-HPLC).

2.3 Preparation of isomerized extracts from supercritical carbon dioxide hops extract

Supercritical CO_2 hops extract containing 41% of α-acids, and 19.5% of β-acids was also used as a raw material for the preparation of other extracts. Those with isomerized α-acids, K/IZ (KHE) and Mg/IZ (MgHE) were prepared from HE extract by the isomerization of α -acids using potassium salts and magnesium oxide, respectively, at the New Chemical Syntheses Institute.

Experiments were carried out in a glass reactor. A schematic diagram of the experimental apparatus is shown in Fig. 1. Hops extract was fed to the glass reactor and slightly heated to temperature of approx. 30°C. Ethyl alcohol (96%) was then added to reduce the extract viscosity. The mixture was homogenized with a stirrer while maintaining a continuous flow of inert gas. After homogenization, a catalyst was introduced and the mixture was stirred for about 5–15 minutes. The reaction mixture was discharged through a drain valve into air-tight containers. Finally the reaction mixture was stored at ambient temperature for several days to allow for the isomerization reaction to complete. The reaction products were analysed with liquid chromatography. The content of iso-α-acids in the isomerized extract was 38% wt.

2.4 Preparation of xanthohumol rich extract from spent hops

Extract rich in xanthohumol (XH4) was prepared from spent hops using supercritical carbon dioxide at pressures up to 100 MPa with a two-stage separation, at the New Chemical Syntheses Institute.

The experiments were carried out in a pilot plant (NATEX, Austria), Fig. 2. This system was equipped with a diaphragm circulation pump (LEWA, type G3S M411), two high-pressure extraction vessels, each of capacity approx. 40 dm^3, two separators for fractionated separation, a condenser, cooling and heating systems. The plant can operate within a pressure range 30–100 MPa, at temperatures up to 100°C.

Spent hops pellets were fed to the extraction vessels and then the vessels were pressurized to 6 MPa. The temperature in the vessels was maintained at 50°C. Pure carbon dioxide (food grade) was compressed by the pump to increase the vessel pressure to 100 MPa. The flow rate of CO_2 was maintained at 200 kg h^{-1} and CO_2 moved upward through the extraction vessel. The product was divided with two separation stages by expanding the stream of CO_2 with the extract at a determined pressure

Figure 2: Pilot plant that was used in this work.

and temperature. Two products resulted and they differed significantly in xanthohumol content. The extract with higher xanthohumol content was obtained in the first separation stage. The extraction products were analyzed using the HPLC. 6.5% XN content fraction was used for further tests.

2.5 Preparation of hops extract by Soxhlet extraction

Beside the supercritical hop extracts, extract prepared by conventional Soxhlet extraction with a non-polar solvent hexan (SOXHE) was also investigated for comparison purposes.

2.6 Materials and reagents

Magnesium oxide (99% of MgO content) and 96% ethanol were purchased from POCH Company Ltd., Poland. Xanthohumol (purity ≥ 96%), as a reference material, was purchased from Sigma, Aldrich, St. Louis, MO, USA. Acetonitrile of HPLC grade was purchased from Avantor Performance Materials, Center Valley, PA , USA.

2.7 Determination of bitter substances in hops extracts

To determine the α-, β-acids and isomerized α- acids contents, an assessment was carried out according to a modified version of the EBC 7.8. (European Brewery Convention) method using U-HPLC.

The reagents for U-HPLC analysis: methyl alcohol of LC/MS purity, acetonitrile of LC/MS purity and trifluoroacetic acid of GC/MS purity were purchased from POCH Company Ltd., Poland; deionised water was produced by the Aquinity E30 water purification system.

In order to carry out the chromatographic analysis, 0.5000 g of the standard or a homogenic extract sample was dissolved in about 30 mL of methanol. The dissolved sample was transferred quantitatively to a 100 mL volumetric flask. The volume was made up to the mark with methanol and mixed. 10 mL of the solution was transferred with a pipette to a 50 mL volumetric flask; the volume was made up to the mark with methanol and mixed thoroughly. Then the solution was filtered through a 0.45 µm membrane filter and 2 µL was injected onto the chromatographic column using separation conditions described above.

The EBC standard was used as an external standard for the calibration of the chromatographic system and for determining of α- and β-acid contents in analysed reaction mixtures. The ICS-13 standard was used for determining the iso-α-acids in the investigated mixtures. The EBC standard (containing 49.4% of α-acids and 24.94% of β-acids) and ICS-I3 standard (containing 62.3% of iso-α-acids) were purchased from Labor Veritas AG, Switzerland.

Hops products were investigated with an U-HPLC chromatograph ACCELA 1250 Thermo Scientific Company with a working pressure of up to 1250 bar and an absorption detector UV-Vis with a diode matrix. A Hypersil Gold column of dimensions 200 × 2.1 nm, grain size 1.9 µm, and pore size 175 A was used as the separation column. The detection of α- and β-acids was carried out at a wavelength of 314 nm, and for iso-α-acids at a wavelength of 270 nm. A binary solvent system: eluent A (content v/v: ultra pure water with 0.1% TFA) and eluent B (content v/v: 90% acetonitrile, 10% ultra pure water and 0.1% TFA) with the following gradient elution: 65% B initially, maintained for 14 min, increased to 100% B in 20 min, decreased to 65% B in 24 min and maintained for 6 min was developed. The used flow rate was of 400 µL min^{-1}.

The total content of iso-α-acids and the total content of α-acids were expressed as a percentage by weight of the sum of the corresponding co-, n- and ad-homologues.

Xanthohumol content in xanthohumol rich extract was determined by UHPLC. Xanthohumol standard (98% purity) was purchased from Sigma Aldrich. Analyses were carried out at 290 nm and 370 nm. A binary gradient elution was applied: A; water + 0.1% of TFA, B: 90% of acetonitrile + 10% of water + 0.1% of TFA. Table 1 presents conditions of the chromatographic separation.

Table 1: A gradient system applied for xanthohumol determination.

Minutes	% A	% B	Flow rate (µl min^{-1})
0	43	57	400
13	43	57	400
15	0	100	400
18	43	43	400
20	43	43	400

2.8 HPLC analysis

The HPLC analysis was performed to determine the xanthohumol content in the investigated extracts. The HPLC fingerprint of the extract and quantification of identified compounds was achieved by HPLC (Agilent Technologies 1200). The detection was performed using a Diode Array Detector (DAD), and the chromatograms were recorded at λ = 360 nm. The HPLC separation of components was achieved using a LiChrospher 100 RP 18e (5 µm), 250 × 4 mm i.d. column, with a flow rate of 1 mL min^{-1} and mobile phase, A [500 mL of H_2O plus 9.8 mL of 85% H_3PO_4 (w/w)], B (MeCN), elution, combination of gradient mode: 80–57% A, 0–5 min; isocratic 57% A, 5–20 min; 57–40% A, 20–35 min; 40–20% A, 35–40min; isocratic, 20% A, 45–50 min; 20–0% A, 50–70min). The sample was prepared by dissolving 53.0 mg of the extract obtained from the procedure previously described in 10 mL of MeOH, filtered through 0.2 µm PTFE filters prior to HPLC analysis. The volume injected was 4 µL. The standard solution for the determination of xanthohumol has a final concentration of 0.01 mg mL^{-1}. To determine the XN content in the investigated extracts, the volume injected was 4 µL, the same as the investigated extract. The compound identification obtained by comparing retention times and spectra matching. Using spectra matching to identify the compounds, the results were confirmed by spiking the sample with the respective standard to achieve a complete identification by means of a peak purity test. Quantification was performed by external calibration with a standard.

2.9 GC-FID and GC-MS analysis

Gas chromatography analysis of the extracts was carried out on a HP-5890 Series II GC apparatus [Hewlett-Packard, Waldbronn (Germany)], equipped with a split–splitless injector and automatic liquid sampler, attached to a HP-5 column (25 m × 0.32 mm, 0.52 µm film thickness) and fitted to a flame ionization detector (FID). The carrier gas flow rate (H2) was 1 mL min^{-1}, the split ratio was 1:5, the injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40 to 260°C (at rate of 8°C min^{-1}), and then kept isothermally at 260°C for 30 min. Solutions of samples in chloroform or alcohol were consecutively injected in amount of 1 µL. The area percent reports, obtained as result of standard processing of chromatograms, were used as a base for the quantification analysis.

The same analytical conditions as those mentioned for GC-FID were employed for GC/MS analysis, while using the column HP-5MS (30 m × 0.25 mm, 0.25 µm film thickness), with a HP G 1800C Series II GCD system [Hewlett-Packard, Palo Alto, CA (USA)]. Helium was used as the carrier gas. The transfer line was heated at 260°C. The mass spectra were acquired in EI mode (70 eV); in a m/z range of 40–450. A sample solution of 0.2 µL in chloroform or alcohol was injected. The components of the extracts were identified by comparing of their mass spectra to those from the Wiley 275 and NIST/NBS libraries, using different search engines. The identification of the compounds were achieved by comparing their retention indices and mass spectra with those found in the literature [18] and supplemented by the Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver. 2.1), GC-MS Libraries [19]. The experimental values for the retention indices were determined by using calibrated Automated Mass Spectral Deconvolution and Identification System Software (AMDIS ver. 2.1), GC-MS Libraries [19], and comparing with those from the available literature (Adams 2007) [18] as well as utilizing an additional tool to approve the MS findings. The relative proportions of the constituents were expressed as percentages obtained by peak area normalization, and all relative response factors were consider as one.

The hops supercritical CO_2 extract sample was analyzed from fatty acids and waxes that were previously separated by precipitation with methanol.

2.10 Antimicroabial activity

For the investigation of antibacterial activity of extracts, the broth microdilution method was applied for determining MIC (minimal inhibitory concentration) values in accordance with the CLSI recommendations (2003) [20]. Investigation of antibacterial activity of the obtained extracts was performed with strains categorized as foodborne pathogens (*L. monocytogenes*, E.coli), food spoilage microorganisms (*B. cereus, S. aureus*) and *Lactobacillus* strains which are part of the

Table 2: The percentage content of α-acids, β-acids, iso-α-acids and xanthohumol achieved by HPLC in the investigated hops extracts: supercritical carbon dioxide extract (HE), extract obtained from HE by isomerization with potassium salts (KHE), extract obtained from HE by isomerization with magnesium oxide (MgHE) and extract obtained after SCO_2 extraction from the spent material, XH4.

Extracts	α-Acids (%)	β-Acids (%)	Iso-α-acids (%)	Xanthohumol (%)
HE	41.0	19.5	-	0.15 ± 0.02
KHE	0.8	23.2	42.9	0.03 ± 0.01
MgHE	0.1	14.4	31.7	1.19 ± 0.04
XH4	0.7	tr	-	6.49 ± 0.11
SOXHE	-	-	-	0.08 ± 0.01

autochthonous cultures in cheese but may also contribute food spoilage processes. Investigated *L. monocytogenes* strains were isolated from brain samples originated from a dead cow and rabbit diagnosed with listeriosis, *E.coli* was isolated from a stool sample originated from adult person with diarrhea, *B. cereus* strains were isolated from a skin swab and from the environment (as contaminants), and lactobacilli was isolated from a cheese specimen. To isolate the bacteria, conventional microbiological methods were applied using Columbia 5% sheep blood agar (bioMerieux, France), MacConkey agar (Becton Dickinson, USA), Tryptic soy yeast extract broth (TSYEB, Biolife, Italy), Difco™ Lactobacilli MRS agar and Difco™ Lactobacilli MRS broth (Becton Dickinson, USA). The commercial system for species identification-BBL Crystal Gram-positive ID System and BBL Crystal Enteric/nonfermenter ID System (Becton Dickinson, USA) were used except for lactobacilli which was not identified to the species level, just genusa. Investigated staphylococci were purchased as referential ATCC strains (Thermo Scientific, USA). To investigate the antibacterial activity of extracts, a broth microdilution method was applied to determine the MIC (minimal inhibitory concentration) values according to the CLSI recommendations (2003). The cation adjusted Mueller Hinton II broth was used (CAMHB, Becton Dickinson) and the antibacterial activity of the extracts was investigated at concentrations of 1280; 640; 320; 160; 80; 40; 20; 10; 5; 2,5; 1,25 and 0,625 μg mL^{-1}. The desired inoculum's density of 5×10^5 CFU mL^{-1} was achieved by preparing a suspension of bacteria of approximately 1–2 × 10^8 CFU mL^{-1}, which was the density equal to the McFarland standard 0.5 (Becton Dickinson). The prepared suspension was diluted 10 times, to obtain a final inoculum density of approximately 1–2 × 10^7 CFU mL^{-1} and 50 μL of this suspension was applied to the CAMHB, the number of bacteria in the media was approximately 5×10^5 mL^{-1}. The media were incubated at 37°C for 18 hours. For MIC values for the broth with the lowest concentration of extract, and no visible bacterial growth, was used.

3 Results and discussion

Contents of α - and beta-acids, as well as iso-α-acids in the investigated extracts, determined by the HPLC analysis, are presented in Table 2. The supercritical CO_2 hops extract (HE) contained 41% of α-acids and 19.5% of β-acids. The KHE contained iso-α-acids (42.9 %), β-acids (23.2%) and α-acids (0.8%), while the MgHE contained iso-a-acids (31.7), β-acids (14.4%) and α-acids (0.1%). The quantification of the acids in sample XH4 revealed the presence of 0.7% of α- acids, whereas β-acids were present in traces, Fig. 3.

The HPLC analysis was performed in order to determine xanthohumol content in the hops extracts (Table 2, Fig. 4). The results indicated that the MgHE extract contained more than 1% of xanthohumol, while in the KHE and HE xanthohumol was present in traces. The content of xanthohumol in the XH4 was determined to be 6.49%.

GC and GC-MS analysis were performed in order to determine the chemical profile of the extracts (Table 3, Fig. 5). GC-MS provides high resolution and the ability to provide precise and accurate qualitative and quantitative data, and as such is a valuable tool for studying plants. Over 170–200 compounds can be separated and their quantities estimated using capillary GC analysis of hops. Both volatile and non-polar compounds can be assessed in one run using capillary GC analysis. This makes this technique a very suitable tool performing comparative study of different samples by chromatographic profiling or fingerprinting. Those results provide phytochemical composition data, which are indispensable for standardization and quality control of plant raw materials, and their extracts of different types required in food or pharmaceutical industry. Besides, the quantification and determination the substances responsible for certain pharmacological properties could provide guidance in choosing a more economic way of obtaining the extracts rich in those substances.

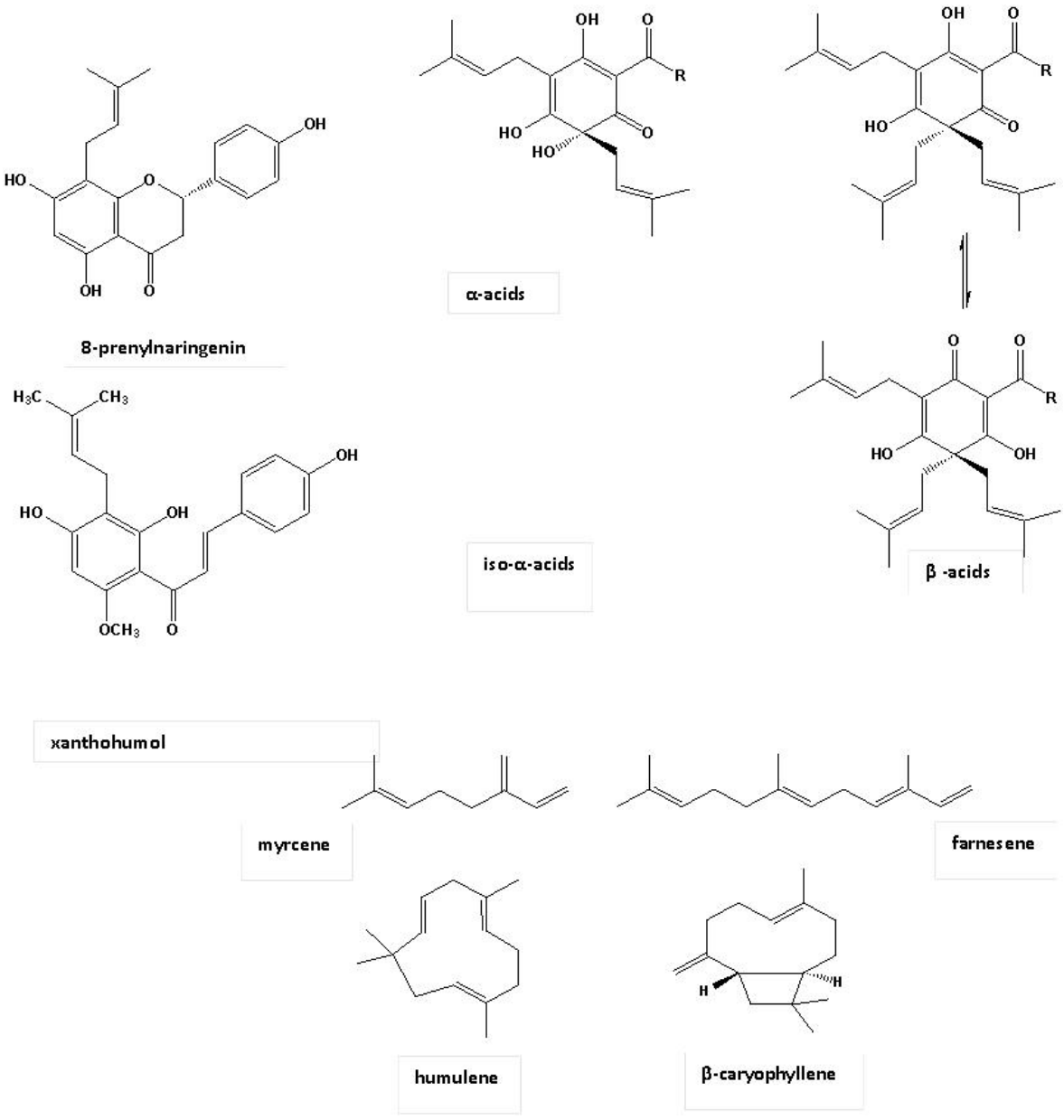

Figure 3: The main constituents of *Humulus lupulus L.,* Cannabaceae *(Hop).*

The constituents in the investigated extracts were analyzed by GC and GC-MS followed by a calculation of the Kovatz indices. In total, 93 compounds were identified (Table 3) in the investigated samples of HE, MgHE, KHE, SOXHE accounting 88.7, 93.5, 82.4 and 78.8% (respectively).

In the HE sample, obtained by SC CO_2 applying the pressure of 30 MPa and temperature of 50°C as extraction conditions, phloroglucinol derivatives and sesquiterpene hydrocarbons were the major constituents (24.8 and 24.7%, respectively), but a significant amount of monoterpene hydrocarbons, oxygenated sesquiterpenes and steroid, diterpene and triterpene fraction were present as well (7.4, 9.5 and 10.7%, respectively). Interestingly, the most abundant compounds were lupulone, α-humulene, (*E*)-β-farnesene, dehydrohumulinic acid, myrcene and *trans*-β-caryophyllene (8.6, 8.2, 7.5, 7.5, 7.1 and 3.6%, respectively).

The significant differences were established between the chemical profiles of the extracts from HE, and SOXHE, obtained by classical Soxhlet extraction with non-polar solvent, hexan. Oxygenated sesquiterpenes, sesquiterpene hydrocarbons and phloroglucinol derivatives were the main

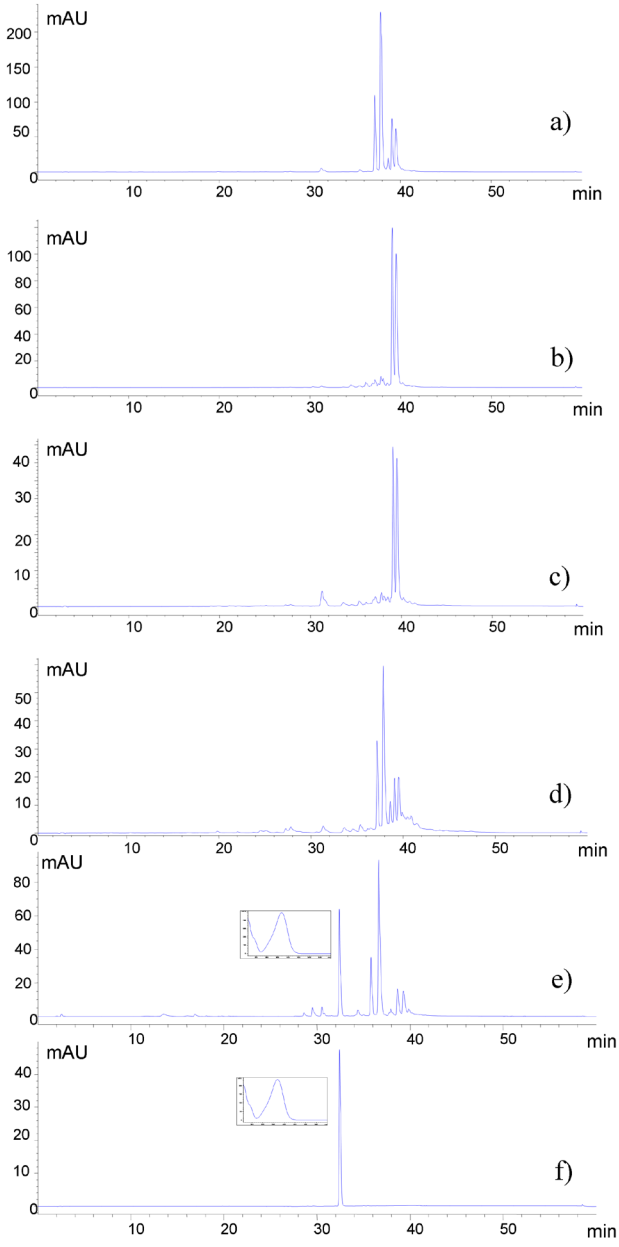

Figure 4: A HPLC chromatogram of the investigated samples HE (a), MgHE (b), KHE (c), SOXHE (d), XH4 (e), and standard xanthohumol (f), recorded at λ = 360 nm. The UV spectra of the compounds in the investigated samples at the retention time corresponding to the standard substance were shown.

compound groups in the SOXHE sample (representing 24.0, 17.6 and 16.2%, respectively). The main identified components were gymnomitrol, dehydrocohumulinic, dehydroisohumulinic and dehydrohumulinic acids, lupulone (6.3 5.4, 3.5, 2.7, 2.2%, respectively). Myrcene and (*E*)-β-farnesene were not identified, while α-humulene and *trans*-β-caryophyllene were present in smaller quantity when compared to the HE sample (3.1 and 1.7%, respectively).

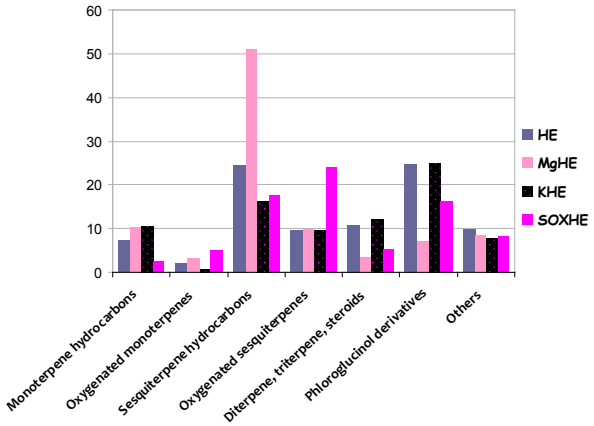

Figure 5: Comparative representation of particular groups of compounds (monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpene, diterpenes, triterpenes and steroids, phloroglucinol derivatives and other compounds) in the investigated extracts.

The sample MgHE obtained by the isomerization of HE, using Mg^{2+} salts was characterized by the presence of sesquiterpene and monoterpene hydrocarbons (50.9 and 10.2%, respectively). The major compounds were α-humulene, (*E*)-β-farnesene, myrcene and *trans*-β-caryophyllene (representing 14.7, 14.1, 10.6 and 6.8% and, respectively), while lupulone was present at only 2.2%. Although the KHE extract had a significant quantity of myrcene and α-humulene (10.6 and 9.4%), the main components was lupulone at 14.7%. This extract also showed the same pattern with the phloroglucinol derivatives content, as the HE, of 25.0%.

Humulone was identified only in the HE sample. *trans*-Isohumulone, a major bitter flavouring component in beer, when exposed to irradiation might undergo of the transformation in several products containing an enolized cyclic β-triketone moiety: *cis*-isohumulone, humulone, *dehydro*-isohumulone, and *dehydro*-humulinic acid, resulting from the loss of the 4-methyl-3-pentenoyl side chain of *trans*-isohumulone [21,22]. In the HE and KHE samples, *trans*-isohumulone and *cis*-isohumulone were identified. The presence of dehydrohumulinic and dehydroisohumulinic acid was detected in the HE, MgHE, KHE and SOXHE extracts, while dehydrocohumulinic was not present in MgHE. Cohulupone is the oxidation product of β-acid colupulone, while hulupone corresponds to lupulone/adlupulone. The presence of hulupone, one of the major oxidized products, as the consequence of high susceptibility of hops to oxygen, was confirmed in the SOXHE sample. In connection with the brewing of beer, hulupons possess the desired bitter taste, but to a much lesser extent than the iso-α-acids. The MS spectra

Table 3: Chemical composition achieved by GC-MS method of the investigated hops extracts.

	Identified compounds	K.I.[a]	HE	MgHE	KHE	SOXHE
1.	isopentanoic acid	832				0.2
2.	propyl caproate ——	927			1	
3.	methyl-pent-2-enolide	945	0.5		0.5	0.4
4.	β-pinene	974	0.3	0.4		0.1
5.	methyl 2-hydroxy-4-methylvalerate	983				0.5
6.	myrcene	988	7.1	9.8	10.6	
7.	butyl butanoate	993				0.9
8.	isoamyl isobutyrate	1007			tr	0.3
9.	β-phellandrene	1025				2.4
10.	isopentyl butanoate	1052	2.4	3.3	1.8	
11.	linalool oxide	1067				0.5
12.	nonanone-2	1087	t			
13.	linalool	1095	0.7	0.8	0.4	1.9
14.	2-methyl isobutyl isovalerate	1103	0.7			
15.	methyl caprylate	1123	2.1	0.4		
16.	*trans*-α-dihydroterpineol	1143				0.6
17.	hexyl butanoate	1191	0.2			
18.	methyl pelargonate	1223	0.5	0.4		
19.	hydroxycitronellal	1286	0.1			
20.	thymol	1289				0.3
21.	undecanol-3	1293	0.4	3.1		
22.	undecanone-2	1293	0.4	0.8	0.4	0.7
23.	carvacrol	1298				1.1
24.	methyl geranate	1322	1.1	2.4	0.4	0.6
25.	methyl-*(Z)*-4-decanoate	1323	0.9	0.6	0.5	
26.	α-cubebene	1345				0.4
27.	dihydroeugenol	1366				0.2
28.	α-ylangene	1373		0.5	0.5	0.5
29.	α-copaene	1374		1.8	1	0.7
30.	β-cubebene	1387				0.4
31.	methyl decyl ketone	1388				0.6
32.	*trans*-β-caryophyllene	1417	3.6	6.8	2.1	1.7
33.	β-cedrene	1419				tr
34.	*trans*-α-bergamotene	1432	1	1.6		
35.	α-humulene	1452	8.2	14.7	9.4	3.1
36.	*(E)*-β-farnesene	1454	7.5	14.1	0.9	
37.	χ-muurolene	1478	1	1.3		1.3
38.	χ-curcumene	1481			0.5	
39.	χ-himachalene	1481		2.4	0.4	

Continued **Table 3:** Chemical composition achieved by GC-MS method of the investigated hops extracts.

	Identified compounds	K.I.[a]	HE	MgHE	KHE	SOXHE
40.	β-selinene	1489	0.5	2.5		0.5
41.	*trans*-muurola-4(14),5-diene	1493				0.3
42.	tridecanone-2	1495			1	0.6
43.	viridiflorene	1496	0.8			
44.	α-muurolene	1500				0.3
45.	*(E, E)*-α-farnesesne	1505	t			
46.	β-bisabolene	1505	t			
47.	garanyl isobuyrate	1514	1.8	2.3	1.3	1.3
48.	χ-cadinene	1522	0.7	2.6	0.6	1.1
49.	δ-cadinene	1522	1	2.6	0.9	
50.	calomenene	1528				1.9
51.	α-calacorene	1542				1.5
52.	β-calacorene	1563				0.6
53.	caryolan-8-ol	1571			0.4	
54.	himachalene epoxide	1578	0.5		0.7	0.8
55.	caryophyllene oxide	1582	0.8	2.1	0.4	1.1
56.	carotol	1594				1.8
57.	humulene epoxide	1603	0.6	0.6	0.4	1.6
58.	1,5,8,8-tetramethyl-cycloundecan-5-ol	1605			1.9	
59.	humulene epoxide (6,7)	1608	1.3	2.5	0.9	1.4
60.	gymnomitrol	1658	2.3	0.7	2.4	6.3
61.	14-hydroxycaryophyllene	1666	1	0.9	0.8	3.4
62.	pentadecen-2-one	1667	0.4		0.3	
63.	*(Z)*-α-santalol	1674				1.6
64.	cadelene	1675	0.4			0.6
65.	14-hydroxy-4,5-dihydro-β-caryophyllene	1706	0.3		0.6	3.3
66.	longifolen	1713				1.6
67.	*(2Z,6E)*-farnesol	1722	0.9	0.8		
68.	sesquilavandulyl acetate	1739				1.4
69.	β-bisabolene	1789				1.1
70.	iso-torquatone demethyl	1844	0.9			1.1
71.	methyl palmitate	1921				1.5
72.	dehydrocohumulinic acid	1933	3		2.2	5.4
73.	palmitic acid	1959	t		0.5	2.1
74.	dehydroisohumulinic acid	1976	1.7	1.2	1.1	3.5
75.	dehydrohumulinic acid	1980	7.5	2.1	4.6	2.7
76.	manoyl oxide	1987				2.4
77.	methyl linoleate	2095			0.4	
78.	methyl α-linolenate	2108			0.6	

Continued **Table 3:** Chemical composition achieved by GC-MS method of the investigated hops extracts.

	Identified compounds	K.I.[a]	HE	MgHE	KHE	SOXHE
79.	ethyl linoleate	2196	0.8		0.7	
80.	methyl squalene	2760	0.7			
81.	*trans*-isohumulone	2811	2		1.4	
82.	*cis*-isohumulone	2813	0.6		1	
83.	humulone	2830	0.5			
84.	6α-acetoxy-4-propyl-3,4-*seco*-5α-androstene-3,17β-diol	2838	5.9	3.6	12.2	
85.	lupulone	2850	8.6	3.8	14.7	2.2
86.	17-hydroxy-5α,17α-pregnan-3-one	2870	2.1			1.1
87.	23-methyl-cholesta-5,22(*Z*)-dien-3β-ol	2897	0.7			
88.	tetrahydrosqualene	2900	0.3			
89.	hulupon	3100				1.3
90.	Brassicasterol	3115	0.2			0.3
91.	(3β,5α,7β,8α,22*E*)-cyclopropyl[7,8]-ergosta-22-en-3-ol-3,7-dihydro	3120	0.2			0.3
92.	23,24-dimethyl-cholesta-5,22(*E*)-dien-3-ol	3283	0.6			1.2
93.	tritriacantone	3300	0.4			0.2
	Total identified compounds		88.7	93.5	82.4	78.8
	Monoterpene hydrocarbons		7.4	10.2	10.6	2.5
	Oxygenated monoterpenes		1.9	3.2	0.8	5.0
	Sesquiterpene hydrocarbons		24.7	50.9	16.3	17.6
	Oxygenated sesquiterpenes		9.5	9.9	9.8	24.0
	Diterpene, triterpene, steroids		10.7	3.6	12.2	5.3
	Phloroglucinol derivatives		24.8	7.1	25.0	16.2
	Others		9.7	8.6	7.7	8.2

[a]K.I. Kovats indices determined experimentally

with the characteristic fragmentation ions permits the identification of the derivatives of humulone and lupulone in the investigated samples [23-25].

The antimicrobial activity of the extracts was investigated against selected strains (Table 4). The results indicated strong antimicrobial activity with MIC values ranging between 2.5–160 μg mL^{-1} (Table 4). The XH samples showed stronger activity against *S. aureus*, in comparison to the other tested extracts. The HE extract had the same MIC as XH for *B. cereus*. *L. monocytogenes* was shown to be especially susceptible to XH, but the activity of the HE was significant, as well. The investigated hops extracts with MIC ≥ 2560 μg mL^{-1} did not affect the *E. coli* from normal intestinal flora, meaning that the oral admission would not cause the same problem as antibiotic application [4]. Our findings are in accordance with the literature data, namely, that XN exibited the most potent inhibition of *S. aureus*, while not inhibiting *E. coli* proliferation, but with MIC value against *S. aureus* determined in our experiment to be lower then stated in the literature (2.5 vs. 6.25 μg mL^{-1}) [26]. The antibacterial activity against *S. aureus* is important because *S. aureus*, due to its adaptability, can easily develop resistance to commonly used antibiotics. This resistance involves the enzyme inactivation in resistant bacteria. Resistant genes are often transferred to other bacteria by a variety of gene transfer mechanisms. Hence, there is a need for an effective antibacterial agent against *S. aureus* with new modes of action. Antibacterial

Table 4: Antimicrobial activity of the investigated hops extracts [supercritical CO_2 ($ScCO_2$) extract (HE); $ScCO_2$ extract isomerized by MgO (MgHE); $ScCO_2$ extract isomerized by KOH (KHE); non-polar hops extract (Sohxlet extraction) SOXHE; xanthohumol (XH, 96%) and $ScCO_2$ extract with XH content up to 6.5% (XH4)], against *L. monocytogenes*, brain specimen, cow; *L. monocytogenes*, brain specimen, rabbit; *B. cereus,* swab from the environment; *B. cereus*, skin swab animal*; Lactobacillus sp*., cheese; *Lactobacillus sp.* cheese; *S. aureus* MRSA ATCC 43300; *S. aureus* ATCC 29213; *Escherichia coli*, adult with diarrhea.

Hop extracts Bacterial strain	(MIC µg/ml) HE	MgHE	KHE	SOXHE	XH	HOPXH4
L.monocytogenes, cow	20	40	20	160	2,5	80
L.monocytogenes, rabbit	10	40	20	160	2,5	80
B. cereus, environment	2,5	40	5	20	20	40
B. cereus, animal	2,5	10	5	20	2,5	20
Lactobacillus sp., cheese	40	320	40	80	1280	160
Lactobacillus sp., cheese	80	320	80	320	1280	2560
S. aureus MRSA ATCC 43300	20	80	40	80	5	80
S. aureus ATCC 29213	20	160	40	80	5	80
E. coli, adult person	>2560	>2560	>2560	>2560	>2560	>2560

agents, originated from natural sources like, XN might be the good choice.

The presence of phloroglucinol derivatives in the investigated extracts, determined by the GC and GC-MS analysis, was in accordance with the literature data, determining that the highest content was in the sample prepared using supercritical CO_2 (HE) and in the isomerized KHE sample. Surprisingly, the content of the phloroglucinol derivatives in the other isomerized extracts (MgHE) was even less in comparison to the extract obtained by Soxhlet extraction (SOXHE) (Table 3 and Fig. 4). This might, at least partly, be an explanation for the smaller antibacterial potential in comparison to rest of the investigated samples. The discrepancy in the bitter acids content between two methods, HPLC and GC-MS might be due to their sensitivity and the degradation that could occur with the analysis method applied.

The hops extracts, obtained by supercritical CO_2 extraction, showed significant antibacterial potential against the investigated bacterial strains. The most potent agent was the XH sample, but significant activity was observed by the supercritical hops HE extract and the extracts obtained by the isomerization procedure, as well, although they do not contain XN. Taking into account the literature data, the hypothesis can be derived that besides XN, α- and β-acids, humulones and lupulones, and the isomerized forms of humulones might be considered as carriers of antimicrobial properties. The possible mechanism of antibacterial activity of bitter acids and their derivatives might include the induced leakage of bacterial membrane cells due to their high hydrophobic properties, especially of lupulone.

It is interesting to point out that the HE and SOXHE samples contained in small quantity the steroid derivatives (Table 3) what might be available to engage in the apoptosis agonist activity. This was confirmed for brassicasterol (ergo-5,22-dien-3β-ol). Of note, it has been reported that intracellular cholesterol accumulation induces apoptosis of pancreatic cells [27].

4 Conclusion

While designing the antimicrobial agents for this study, the main factor, which could influence the choice of the applied extract, should be the fact that it does not affect the taste, odor and appearance of the food in the doses applied. Low MIC values obtained in this study are promising for the application of hops extracts in various types of food products (frozen meals, fish and meat products, juices) with no side effects on the organoleptic properties of the product. The investigated hops extracts did not affect the *E. coli* from normal intestinal flora, meaning that their oral admission would not cause the same problem as antibiotic application.

The primary aim of this study was to present the differences in antibacterial activity of different hops extracts obtained using the supercritical carbon dioxide extraction, and afterwards by applying the processes of modifying the base extract in order to obtain the extracts

that might be used in brewery industry. The antiseptic properties and the soft-resin contents of the hops are not directly proportional to each other, and therefore estimations of the soft resins do not constitute accurate measures of the antiseptic properties of hops. Also, special attention has been paid to the extracts obtained from the spent hops cones.

Acknowledgements: This project was partially financed in the framework of grant **PBS1/A5/18/2012** entitled: "Development of the new generation and ecological and safe in use cosmetics and products of the household chemistry based on the plant supercritical extracts" attributed by the National Center for Research and Development.

This work was also partially financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project III45017).

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