FLAVONOLS HPLC ANALYSIS, IN VITRO BIOLOGICAL ACTIVITIES IN SELECTED *Humulus lupulus* L. GENOTYPES

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Abstract: The *Humulus lupulus* L. is well known as necessary raw material for beer production. The main structural classes of chemical compounds identified from hop cones include terpenes, bitter acids, prenylated chalcones, and flavonol glycosides. They were subjects of presented work. The content of quercetin was found in the range 490 – 1092 µg/g and that of kaempferol from 218 to 568 µg/g of the dry hop cones. The content of isorhamnetin was very low in all varieties. From biological activities in vitro point of view, relative high level of inhibition activity was observed for six hop genotypes – Zlatan, Lučan, and the Oswald's clones 31, 70, 71, 72, 114 on both enzymes thrombin and urokinase, but without correlation to analyzed flavonols content. In spite of this, antioxidant activity, measured by both the BCLM and HPE methods, was found high and seem to be in correlation with content of analyzed flavonols. Particularly the Oswald's clone 114 expressed very potent biological activities. In general, obtained results indicate that hop cones are valuable material also for other application others than beer production.

Key words: *Humulus lupulus*, Saaz, quercetin, kaempferol, isorhamnetin, biological activity in vitro

1. Introduction

The hop plant (*Humulus lupulus* L., *Cannabinaceae*) is well-known throughout the world as the raw material for brewing industry. The female inflorescences (the hop cones, *Strobili lupuli*) are used in the beer production especially to add bitterness (STEVANS et al., 1999). The main structural classes of compounds identified from the hop cones include: terpenes (compounds with sedative effect), bitter acids (prenylated derivatives of phloroglucinol, which posses antibacterial and antioxidant effects) and finally polyphenols, including flavonoids, prenylated flavonoids, and polyphenolic acids with antioxidant, antibacterial activity (KROFTA et al., 2008; ZANOLI and ZAVATTI, 2008; NESVADBA and KROFTA, 2009).

It is well now that flavonoids exhibit a broad spectrum of pharmacological effects, especially anti-inflammatory and antioxidant activity, antimutagenic and anticarcinogenic activities but also inhibition activity on various proteolytic enzymes including enzymes involved in coagulation and fibrin clot digestion processes as thrombin and urokinase are (MIDDLETON et al., 2000; MALIAR et al., 2004).

Thrombin is key enzyme in process of fibrin clot formation of the coagulation cascade mechanism, partially different from the trypsin by the specific P1' region
cleaving peptide bond between arginine and any bulky aromatic residue (ONAYA et al., 1998). Thrombin is pathophysiological promotor of coagulation disorder diseases and attractive target for perspective thrombolytics.

Urokinase (plasminogen activator of the urokinase type) is key enzyme responsible for activation of plasmin cascade leading to digestion of needless fibrin clot after wound or vessel closing and healing. Urokinase is very specific serine-proteinase preferring cleaving peptide bonds beside lysine residue (SCHMITT et al., 1997), pathophysiological promotor of the oncological diseases with significant metastasis and attractive target for new oncolytics by the metastasis blocking mechanism.

The hop is rich for flavonolglycosides, which have mono- or di-saccharide bonded in position 3. Segawa et al. have identified quercetin glycosides and kaempferol glycosides in the hop cones by mass spectrometry (SEGAWA et al., 2006). The flavonolglycosides hydrolysis release free aglycons – flavonols from mono- or di-sacharides (Fig. 1). Quercetin is formed from the glycosides of rutin, isoquercitrin and isoquercitrin malonate under acidic condition. Kaempferol is formed from similar glycosides – kaempferol rutinosid, astragalone and astragalin malonate. Isorhamnetin was also identified in the hop cones after enzyme hydrolysis (WOJDYLO et al., 2007). During brewing, the flavonols are extracted from hop to beer. The final content of flavonols in beer depends on both, the raw material and the brewing process conditions.

However, acid hydrolysis takes place most frequently (ALEKSEEVA et al., 2004; PROESTOS et al., 2006; STALIKAS, 2007). Simultaneous acid hydrolysis and extraction of flavonols was firstly described by Hertog for fresh and frozen vegetables (HERTOG et al., 1992). Additional options are microwave assisted acid hydrolysis (NUUTILA, 2005) or enzyme hydrolysis by glycosidases (WOJDYLO et al., 2007).

Fig. 1. Structures of quercetinglycosides and their acid hydrolysis product.
Physical and chemical properties of flavonols provide important knowledge for selection of the condition for hydrolysis, separation and detection. 1-octanol/water partition coefficient – logP values and solubility in water are given in the Table 1.

<table>
<thead>
<tr>
<th>Flavonol</th>
<th>logP</th>
<th>Solubility in water</th>
<th>Flavonol</th>
<th>logP</th>
<th>Solubility in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>0.21</td>
<td>0.15</td>
<td>Quercetin</td>
<td>1.82 ± 0.32</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.125</td>
<td></td>
<td></td>
<td>0.06 (16 °C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.54</td>
<td></td>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>3.11 ± 0.54</td>
<td>1.99</td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td></td>
<td></td>
<td>not available</td>
</tr>
</tbody>
</table>

Despite of the fact, that these values are available by various chemical software, experimental data are most precious (ROTHWELL et al., 2005). Solubility of aglycones in water is lower than the solubility of glycosides therefore it is necessary to perform hydrolysis of glycosides in water-alcohol solutions.

Saaz is the former German name of the Czech town Žatec, known by the hop cultivation. Nevertheless, it denotes also a hop variety, classified as one of the four true noble varieties used in production of pilsner style beer. This semi-early red-bine hop, belonging to the old middle European aroma group of hops, originates from the clonal selection in original hops widespread within the Saaz and Auscha regions. Saaz variety is grown in nine clones. Its typical property is fine balanced bitterness and the high level of total polyphenols. In fact, in the literature, does not existed sufficient analysis results about flavonols content, especially for these hop genotypes, typical for mentioned region, as well as any indication to biological effect is missing.

The aim of this work was firstly: investigation of the content of individual flavonols in the hop clones Saaz, secondly – estimation of several biological activities on in vitro level, eventually specification of any relation between flavonols content and biological activity. For the first purpose the HPLC analysis was selected and three steps were necessary to accomplish this work: (1) Selection of optimal conditions of the glycoside hydrolysis and extraction of flavonols. (2) Selection of the optimal mobile phase and the most suitable column with the stationary phase. (3) Description of the basic validation characteristics of the flavonols determination. (4) Elaboration of analysis of natural samples. Secondly, selected assays on in vitro level, carried out with the same hop cone extract samples, aimed to attempt to formulate any relations.

### 2. Materials and methods

#### 2.1 Chemicals

Methanol (HPLC gradient grade) was purchased from Sigma-Aldrich. Hydrochloric acid and formic acid (both p.a. purity) were purchased from Mikrochem. Ultra pure water was prepared with Simplicity 185, Millipore. Standards for HPLC: quercetin, kaempferol and isorhamnetin were purchased from Sigma-Aldrich.
2.2 Calculation of physico-chemical properties

The log $P$ values were calculated by ALOGPS 2.1 software accessible on-line on internet (VCCL, 2011).

2.3 Plant material

Plant material of dried hop cones was provided by the Research Institute of Plant Production Piešťany, Slovak Republic and Hop Research Institute Žatec, Czech Republic.

2.4 HPLC analysis

The chromatographic apparatus consisted of a Model 1525 Binary HPLC Pump, a Model 2487 Dual $\lambda$ Absorbance Detector, a Model 2707 Autosampler, a Model 1500 Heather column, and software Empower 2 (Waters, Milford, MA, USA). The optimization of HPLC conditions of flavonoids determination in hop extract was performed with the following colons: Symmetry® C18, 4.6 x 75 mm, 3.5 $\mu$m, X Terra® MS C18, 4.6 x 30 mm, 2.5 $\mu$m, X Bridge™ C18, 4.6 x 50 mm, 3.5 $\mu$m (Waters, Milford, MA, USA). The mobile phase was composed from methanol and ultra-pure water with 0.1 % formic acid. The wavelength 360 nm was selected for detection of all investigated flavonols (quercetin, kaempferol and isorhamnetin).

2.5 Enzyme inhibition assays

Analyses of thrombin, and urokinase inhibition activity in hop cones extracts was determined by simple photometry method using microplate reader OPSYS (Dynex, USA), with following chromogenic substrates: N-$\alpha$-benzoyl-phenylalanyl-valyl-arginine-paranitroanilide (BPVA-pNA) for thrombin, and N-glycine-arginine-paranitroanilide dihydrochloride (GAPNA.HCl) for urokinase, respectively. Substrates were cleaved by thrombin and urokinase according to described methods (ERLANGER et al., 1961; GEIGER et al., 1991), released free paranitroaniline was detected at wavelength 410 nm. Microplates were prepared manually by gradual dissolution of the substrate -inhibitor mixture. Each well (three parallel wells were used for each sample) contained buffer solution with 0.6 mM substrate, 1% DMSO (v/v), and tested sample diluted 200 times. Control wells contained DMSO. Reaction was started by adding of enzyme solution (thrombin – 150 NIH units.mg$^{-1}$, urokinase – 500 Plough units, respectively) in TRIS-HCl buffer, pH 7.6, without Ca$^{2+}$ ions and other activators. Reaction temperature was 25 °C, data scanning time 1 min and 61 min. The optical density difference $\Delta$OD (OD$_{61,st.min}$ – OD$_{1,st.min}$) was measured for each sample. The percentage expression of the inhibition activity was calculated in according with equation (eq. 1) and then related to standard epigallocatechin gallate (EGCG) and expressed in mg of EGCG equivalent (EGCGeq.).

\[
\% \text{ inhibition activity} = ((1 – (\Delta \text{OD sample}/\Delta \text{OD control})) \times 100)
\]  

(1)
2.6 Antioxidant assays

BCLM (β-carotene linoleate model) is simple model of antioxidant activity in vitro (OHSHIMA et al., 1998). This method quantifies ability of the sample to prevent lipoperoxidation under lipophilic conditions. 2 mg of β-carotene were dissolved in 20 mL of chloroform. 4 mL of this solution were added to the evaporation bank with 40 mg of linoleic acid and 400 mg of Tween-40. Chloroform was completely evaporated using rotary vacuum evaporator at 50°C and residue was mixed with 100 mL of deionized water to produce β-carotene linoleate emulsion. 230 µL of this emulsion were mixed with 20 µL of tested extract sample in microplate wells. Microplates were incubated at 50°C for 90 minutes and the reaction mixtures were measured on β-carotene content decrease at 490 nm. Control wells contained pure water and standard wells contained, 90 mM solution of EGCG in DMSO. This method of antioxidant activity was carried out to use an endpoint photometric measurement of a dual wavelengths mode by using a microplate reader type MRX (Dynex). Measured data were processed by same way and related to standard epigallocatechin gallate (EGCG) and expressed in mg of EGCG equivalent (EGCGeq.).

The second method of determination of antioxidant activity was method of hydrogen peroxide elimination (HPE), based upon the catalytical effect of horse radish peroxidase with combination with fenol red in according to method described by PICK and KEISARI (1980) modified by RAKOTOARISON et al. (1997). 10 µL of tested extract sample was mixed with 10 µL H2O2 and immediately filled to 100 µL by phosphate buffer PBS (pH = 7.4). Prepared reaction mixture was subjected to incubation for 15 minutes in thermostat under 37°C. After incubation 100 µL of fenol red solution (0.2 mg/1mL) contained horse radish peroxidase (17 U/1mL) was added and then incubated 15 minutes under room temperature. The reaction is terminated by addition 5 µL 1N NaOH (1 N) and OD 630nm was detected by microplate reader. Measured data were processed by mentioned way and related to standard epigallocatechin gallate (EGCG) and expressed in mg of EGCG equivalent (EGCGeq.).

2.7 Antimicrobial activity

Extract samples were tested using microplate dilution method using sterile microplates with U shape wells. Microplate preparing: filling columns 2, 3, 4, 5, 6, 7, 8, 9, 10 with 100 µL of cultivation medium with inoculum. Then 180 µL of this mixture was added to the column 1, together with 20 µL of tested extract samples. Then is carried out the dilution by gradual transfer of 100 µL z from the wells of column 1 to the column 2, mixing then to column 3, 4 ....12. In the columns there are following titer values of tested samples (from the column 1 to column 12): 10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120, 10240. Cultivation medium was nutrient broth with inoculum adapt to 0.04 – 0.06 McFarland units, for yeast and molds was used malt broth with inoculum adapt to 105 cell per 1 mL using Bürker chamber. Microplates were cultivated under following conditions: bacterial species for 24 hours
at 37°C, yeasts 24 – 48 hours at 22°C (24 – 48 hours), and molds 114 hours at 22°C. Thirty microliters of 0.02% tetrazolium blue (TTB) solution were added after cultivation and after 30 minutes of incubation under same conditions were microplates measured and evaluate. Blue formazane product released by microorganism mitochondrial dehydrogenase has been observed in wells with cell proliferation. The last well without color effect is value of MIC parameter expressed in titer scale.

3. Results and discussion

3.1 HPLC analyze of flavonols in hop cone extracts

At first, the simultaneous acid hydrolysis and extraction of flavonols was optimized with regard to concentration of hydrochloric acid, hydrolysis time, and proportion of methanol in the solution (Fig. 2). The optimized conditions of hydrolysis of the hop cones are as follows: 1.2 mol/L solution of hydrochloric acid during 1 hour hydrolysis in 50 % methanol-water solution.

![Graph](image)

Fig. 2. Optimization of the simultaneous acid hydrolysis and extraction of flavonols from hop cones conditions, (♦) quercetin, (■) kaempferol. Relation of the retention factor \( k \) of flavonols to methanol portion (%) in mobile phase, (♦) quercetin, (■) kaempferol, (▲) isorhamnetin.

A good separation was achieved if the composition of mobile phase was 40 – 45 % methanol in water with addition of 0.1 % formic acid. Further decrease of the methanol content leads to prolongation of the total analysis time. Consequently, the best conditions for HPLC separation of flavonols are as follows: mobile phase 0.1% (w/v) formic acid in water and methanol (57:43), flow rate 1 mL/min, column temperature 45°C. Under these conditions and using the optimized stationary phase (discussed in the next paragraph) the total analysis time is 6 min.
Different stationary phases were compared in the final step to obtain the best resolution of the analyzed compounds. When using the columns Symmetry and X Terra a shift of the retention time $t_R$ of the analytes was observed. The shift of the retention time did not appear when the column X Bridge was used.

The HPLC chromatogram obtained with the mentioned best column, optimally selected mobile phase and further optimized conditions is demonstrated on Fig. 3.

![Fig. 3. Representative HPLC chromatogram of a real extract sample: Humulus lupulus Oswald clone 114. Denotation of peaks: I - quercetin, II - kaempferol, isorhamnetin - detected only in traces. Column XBridge, mobile phase 0.1 % formic acid in water/methanol (57:43).](image)

Basic validation characteristics of the HPLC flavonols determination – calibration equation, linear range, coefficient of determination, LOD and LOQ are summarized in Table 2.

<table>
<thead>
<tr>
<th>Flavonols</th>
<th>Calibration equation $^a$</th>
<th>Linear range [μg/mL]</th>
<th>$R^2$ $^b$</th>
<th>LOD $^c$ [μg/mL]</th>
<th>LOQ $^c$ [μg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>$y = 17275x - 960.3$</td>
<td>0.134 – 8.16</td>
<td>0.9997</td>
<td>0.0726</td>
<td>0.090</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>$y = 18287x - 636.1$</td>
<td>0.0437 – 3.18</td>
<td>0.9997</td>
<td>0.0509</td>
<td>0.0745</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>$y = 14955x + 43.94$</td>
<td>0.025 – 0.100</td>
<td>0.9937</td>
<td>0.0272</td>
<td>0.0572</td>
</tr>
</tbody>
</table>

$^a$ $y$ = peak area, $x$ = concentration of standard in μg/mL.

$^b$ $R^2$ – coefficient of determination for 8 data points in calibration curves. Two parallel measurements were made for each calibration standard.

$^c$ LOD – limit of detection, LOQ – limit of quantification.

Before HPLC analysis of the particular hop plant material it is important to verify the stability of three investigated important flavonols in the solution during hydrolysis. Therefore the solutions of flavonols were subjected to model hydrolysis (reflux, 1.2 mol/L HCl in 50 % water/methanol solution for 2 hours). The model solutions were analyzed in 15 min intervals. Flavonols concentration was not significantly
changed during 60 min, however, after 105 min a significant decrease of the concentration of all three flavonols was observed (not presented in this paper).

In the application part of this study seven varieties of Saaz, harvested during year 2009, were analyzed: Zlatan, Lučan, and the Oswald clones 31, 70, 71, 72, 114 (Table 3). The content of quercetin was found in the range 489 – 1092 µg/g and that of kaempferol 218 – 568 µg/g of the dry hop cones. The content of isorhamnetin was very low in all varieties, which caused a relatively high RSD values. It is worth to compare that the similar quercetin content was found in the hop cones of variety „Sbornyi“ – 1000 µg/g of the dry hop matter (ALEKSEEVA et al., 2004). To discuss about obtained results it is very difficult because there are no published papers about analysis of hop cone from Žatec region, moreover there are several papers aimed to identification of flavonols by MS without quantification (MAGALHÃES et al., 2010).

Table 3. Contents of flavonols and biological activity of dry hop cones.

<table>
<thead>
<tr>
<th>Flavonols</th>
<th>Concentration of the Saaz clones of <em>Humulus lupulus</em> L. in [µg/g] ± % SD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lučan</td>
</tr>
<tr>
<td>Quercetin HPLC</td>
<td>783.3 ± 2.9</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>284.8 ± 4.0</td>
</tr>
<tr>
<td>Isorhamnetin HPLC</td>
<td>traces</td>
</tr>
</tbody>
</table>

3.2 Biological assays of hop cone extracts in vitro

The same sample of extract from hop cones were subjected to several assays in vitro, especially to two test of antioxidant activity, either test of inhibition activity on thrombin as potential pathophysiological promoter of cardiovascular coagulation diseases or test of inhibition activity to urokinase as expression of ability to suppress processes of metastasis and onco-transformed cell spreading. Fig. 4 presents mentioned biological activities of six hop cones extracts (Zlatan, Lučan, Oswald's clones 31, 70, 71, 72, 114).

There is evident (Fig. 4) relative high level of inhibition activity on both enzymes, higher on thrombin for Lučan, Zlatan and Oswald's clones K-70, 71, and for urokinase in case of K-72 and K-114. These indicated certain level of ability to block undesired processes of coagulation cloth formation and extracellular matrix degradation in relation to cardio-vascular diseases or metastasis formation, respectively. These results do not correlate with amount of estimated flavonols what could be explained by the in vitro effect of other active compounds – extractives from hop cones – bitter acids, terpens and others. It was published that the 8-prenylarigenin (8-PN) as one component of hop extract inhibits platelet aggregation induced by thrombin (DI VITO
et al., 2012) but comparison to complex hop cones extract it is not possible, not published yet.

![Graph showing inhibition activities on thrombin and urokinase](image)

Fig. 4. Inhibition activities on thrombin (IA_Thrombin), urokinase (IA_Urokinase), expressed by EGCG equivalent in mg/g of native matter, further antioxidant activities by BCLM method (AOX_BCLM) and by HPE method (AOX_HPT), expressed as TROLOX equivalent in mg/g of native matter for six different hop genotypes. All tested categories are on the level of statistical significance with p<0,1 to control.

Opposite this, antioxidant of hop, hop extract, hop cones were published relatively frequently (LERMUSIEAU et al., 2001; YAMAGUCHI et al., 2009; TRONINA et al., 2013), but differences in applied methods by various labs does not allow systematic comparison. Similarly, level of the antioxidant activity is relative high, but intensively varied between tested samples, the highest for genotype K-114 and the lowest level has been observed for genotype K-71, what indicates rough correlation to flavonoid content. Concerning to obtained results flavonoids seem to be major compounds of hop cone extracts responsible for antioxidant effect by both used methods.

Image about biological activity of hop cones extract was completed by test of antimicrobial activity towards G+ bacterial species *Staphylococcus aureus*, G- bacterial species *Pseudomonas aeruginosa* and *Escherichia coli*, yeast *Candida albicans* and two cereal fungal pathogens *Fusarium graminearum* and *Pyrenophora teres*. The results presents following Table 4.

From the results it is evident high level of antimicrobial activity to cereal pathogen *Pyrenophora teres*, and relatively low activity to clinical bacterial pathogens and yeast with myceliar form *Candida albicans*. From the comparison point of view between tested hop cones extract samples are statistically not significant differences. In fact, several papers published antibacterial effect of hop extracts (NATARAJAN et al., 2008; LEITE et al. 2013) but certain level of antibacterial effect could be explained by flavonols too (WAAGE and HEDIN, 1985; SIVASOTHY et al., 2013).
Generally we can conclude that hop cones and preparations from them are valuable raw material with significant biological activities expressed in vitro.

Table 4. Antimicrobial activities of six hop cones extract samples to selected microbial species.

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<thead>
<tr>
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<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>40</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td><em>Fusarium graminearum</em></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Pyrenophora teres</em></td>
<td>80</td>
<td>160</td>
<td>20</td>
<td>20</td>
<td>80</td>
<td>160</td>
<td>20</td>
</tr>
</tbody>
</table>

4. Conclusions

The RP-HPLC method reported here represents a simple and rapid technique for simultaneous determination of important flavonol aglycones as quercetin, kaempferol and isorhamnetin contained in the hop cone extracts. For seven tested Saaz clones, this is the first report regarding the conditions of the HPLC determination of flavonols. The highest content of quercetin and kaempferol was determined in Oswald's clone 114. In general, obtained results indicate surprisingly high degree of biological activities expressed in vitro and finally conclusion that hop cones are valuable material for other applications, others than beer production.

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References


NESVADBA, V., KROFTA, K: Variability in the content of important compounds for pharmaceutical and brewing industries within hopgene pool. Agriculture (Poľnohospodársťv) 55, 2009: 10-16.


