Plant Cell, Tissue and Organ Culture (PCTOC) (2023) 153:295–306
https://doi.org/10.1007/s11240-023-02462-1

ORIGINAL ARTICLE

Vitro-derived hop (*Humulus lupulus* L.) leaves and roots as source of bioactive compounds: antioxidant activity and polyphenolic profile

Benedetta Chiancone[1] · Valeria Guarrasi[2] · Leandra Leto[1] · Lorenzo Del Vecchio[1] · Luca Calani[1] · Tommaso Ganino[1] · Martina Galaverni[1] · Martina Cirlini[1]

Received: 31 July 2022 / Accepted: 31 January 2023 / Published online: 10 February 2023

Abstract

Bioactive compounds extracted from plant are of great value for those enterprises interested in the use of natural products; plant tissue culture techniques guarantee a reliable and constant biomass production. Hop (*Humulus lupulus* L.), with its wealth in bioactive compounds, may represent an invaluable resource. The present study focused on the characterization of *vitro*-derived leaves and roots of two hop plant types, Cascade and Gianni. Extracts obtained from the selected hop explants were investigated, determining their polyphenolic content as their antioxidant capacity, applying DPPH, ABTS and FRAP assays; moreover, extract molecular profile was obtained through UHPLC-MS/MS. Results confirm the wealthy in bioactive compounds and the antioxidant properties of the tested *vitro*-derived hop explants. The qualitative characterization of *vitro*-derived hop tissue extracts evidenced the presence of twenty one different compounds, already identified in open field grown hop plants, such as polyphenols, α- and β-acids, as well as xanthohumol and isoxanthohumol. The obtained outcomes lay the groundwork to further investigate the potential of *vitro*-derived hop plantlets as bioactive compounds source.

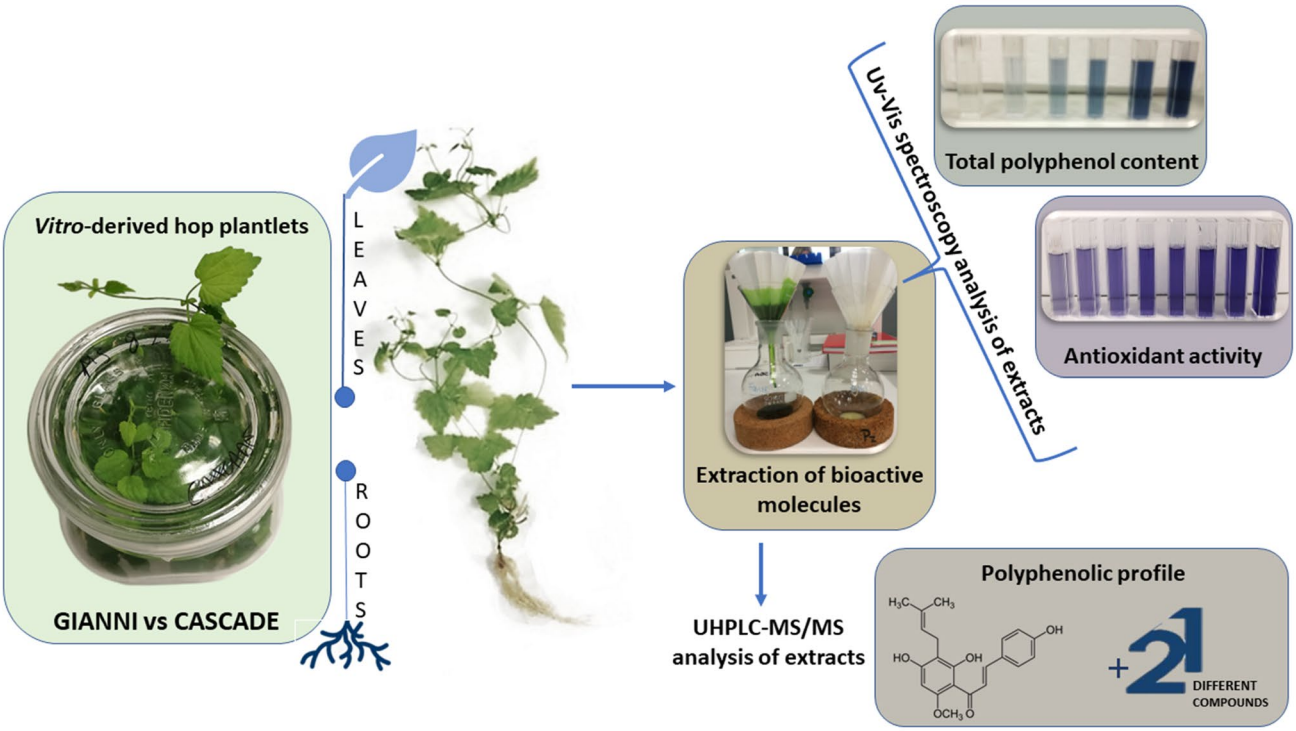
Communicated by Christophe Hano.

✉ Benedetta Chiancone
benedetta.chiancone@unipr.it

[1] Department of Food and Drug, University of Parma, Viale Parco Area delle Scienze 27/A, 43124 Parma, Italy

[2] Institute of Biophysics, National Research Council (CNR), Via Ugo La Malfa 153, 90146 Palermo, Italy

Graphical abstract



Key message

Characterizing and recovering secondary metabolites from micropropagated hop plantlets.

Keywords *Humulus lupulus* L. · Polyphenolic content · Polyphenolic profile · UHPLC-MS/MS · *Vitro*-derived plants

Introduction

Natural products, derived from an increasing range of different plants, are drawing the attention of an everyday wider portion of enterprises, interested in application of plant-based additives in food, pharmaceutical, and cosmetic sector. In this contest, hop (*Humulus lupulus* L.), with its wealth in bioactive compounds, is an invaluable candidate to be exploited (Astray et al. 2020; Kramer et al. 2015; Nionelli et al. 2018; Sanz et al. 2019).

Hop is cultivated all around the world, and, lately, also in Italy, for its cones, key ingredient for beer production, but also historically used in traditional and folk medicine against several diseases (Alonso-Esteban et al. 2019; Liu et al. 2015). Hop bioactive properties are due to the presence of numerous secondary metabolites, including terpenoids, phenolic compounds, alkaloids and bitter acids, related to antioxidant, antimicrobial and antiviral capacities (Alonso-Esteban et al. 2019; Hrnčič et al. 2019; Karabín et al. 2016; Krottenthaler 2009; Roehrer et al. 2019). In particular, the main secondary metabolites identified in hop pertain to different chemical classes as polyphenols (catechin, epicatechin, rutin, coumarin, gentisic acid, caffeic acid, ferulic acid, sinapic acid, quercetin, etc.), α- and β-bitter acids (humulone, cohumulone, lupulone, colupulone), and terpenoids (β-myrcene, caryophyllene, humulene, β-farnesene, α- and β-selinene) (Bocquet et al. 2018; Nezi et al. 2022) (Table 1). All these compounds are mainly secreted by lupulin glands (Stevens et al. 1997), and accumulated at high concentrations in glandular trichomes of cones, but also detected in the vegetative biomass, such as leaves and stems (Gerhäuser 2005; Kavalier et al. 2011). As stated by Čeh et al. (2007), leaves, today considered a waste, may indeed represent a remarkable bioactive compound resource. On the other hand, the types and amount of bioactive compounds recovered by the vegetative hop biomass could be variable depending on the season, because plant secondary metabolism may be influenced by several factors, as environmental conditions (Abram et al. 2015; Green 1997). Furthermore, the accumulation of polyphenols, α- and β-acids, and xanthohumol in the different plant compartments can be cultivar dependent, as described by Čeh et al. in 2007.

Table 1 Hop secondary metabolites and their related bioactivities

Hop secondary metabolites	Bioactivity	References
Terpenoids Myrcene, β-Caryophyllene, Humulene, β-Pinene, Linalool, Limonene, α- and γ-Terpinene, Geraniol, α-Bisabolol, α-, β- and γ-Bisabolene, β-elemene, Fenchone, Pulegone, α-Phellandrene, β-eudesmol *Other terpenes and derivatives:* Isopulegol, Isoborneol, Sabinene, D-cadinene, Farnesol, Farnesene, Myrcenol, β-Ocimene, Linalyl acetate, γ-elemene, p-Cymene Lupeol, α-amyrin	Anti-inflammatory, antioxidant, analgesic, anticonvulsive, antidepressant, anxiolytic, anticancer, antitumor, neuroprotective, antimutagenic, anti-allergic, antibiotic and anti-diabetic properties	Nuutinen (2018)
Phenolic compounds *Chalcones*: Xanthohumol, desmethylxanthohumol *Flavanones*: Isoxanthohumol, naringenin, (±)-8-prenylnaringenin *Flavonols and their glycoside*: Quercetin *Flavan-3-ols*:Catechin, Epicatechin	Anti-leukemic and antiplatelet activities, antimicrobial properties, estrogenic properties, sedative-like activity, antioxidant and anti-infective properties, anti-proliferative, antibacterial, antifungal and antiviral activities	Benelli et al. (2012), Chadwick et al. (2006), Delmulle et al. (2006), Kitaoka et al. (1998), Lee et al. (2012), Milligan et al. (1999), Milligan et al. (2000), Srinivasan et al. (2004), Tabata et al. (1997), Tapiero et al. (2002), Yong et al. (2015), and Zanoli and Zavatti 2008)
Bitter acids or Acylphloroglucinols *α-acids*: humulone, co-humulone, ad-humulone *β-acids*: lupulone, co-lupulone, ad-lupulone	Antimicrobial potential, anticancer, anti-inflammatory, antibacterial, antifungal and antiviral activities	Bhattacharya et al. (2003), Bocquet et al. (2018), Haas and Barsoumian 1994), Hall et al. (2008), Siragusa et al. (2008), Srinivasan et al. (2004), Tyrrell et al. (2010), Tyrrell et al. (2012), and Van Cleemput et al. (2009)

The application of in vitro culture techniques could bypass the differences in the secondary metabolite production caused by cultivation conditions. The United Nations for Food and Agriculture (FAO) recognised since 1994 in vitro plant tissue culture techniques as an important tool for the large-scale production of bioactive compounds (Anand 2010; Dal Toso and Melandri 2011; Dias et al. 2016; FAO 2002; Rao and Ravishankar 2002). It is, indeed, well acknowledged that *vitro*-derived plant organs synthetize the same bioactive compounds produced in corresponding in field grown plants (Scarpa et al. 2022), with the added value of a controlled growing environment that guarantees a more reliable and constant production; in addition, in vitro biomass production is continuous and independent of the season, important detail for deciduous species, like hop. Moreover, in vitro plant tissue culture provides the indispensable stress that triggers the synthesis of secondary metabolites, and specifically of polyphenols, in plants (Buchanan and Jones 2000).

This study aimed at evaluating *vitro*-derived hop leaves and roots as source of bioactive compounds, through the characterization of their polyphenolic and antioxidant fractions. To this goal, Folin–Ciocalteau, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays were applied, and UHPLC-ESI-MS^n technique was used to identify the large part of extract components.

Material and methods

Plant material

Explants destined to extraction were isolated from in vitro cultured hop plantlets of two plant types, the variety Cascade and the ecotype Gianni. Cascade is an aromatic variety selected in 1972, from an unknown American variety, Fuggle and Serebriankermthat, that has become one of the most cultivated all over the world, included several Italian regions (Santagostini et al. 2020); actually, this variety is characterized by a high yield and tolerance to several diseases, and used by the brewer industry for the floral and fruity aroma of its cones (Rodolfi et al. 2019; Santagostini et al. 2020). Gianni is a wild hop ecotype recovered in Emilia Romagna (Northern Italy), and grown in a collection field in Marano sul Panaro (Modena, Italy); over the last years, this ecotype has been phytochemically and morphologically characterized revealing its numerous positive technological attitudes, among which its dual purpose (Mongelli et al. 2015). Both plant types were in vitro established and cultured in 500 ml glass jars, containing the culture medium with the following composition: Murashige and Skoog (MS) basal salt mixture (1x) (Murashige and Skoog 1962), MS vitamin mixture (1x) (Murashige and Skoog 1962), 30 g/l of sucrose and 8.0 g/l of Agar. Culture medium, after adjusting the pH to 5.8 with 1N NaOH, was sterilized in autoclave for 20 min at 121 °C. Glass jars with Cascade and Gianni plantlets were then

sealed, and maintained in a growth chamber at 25 ± 1 °C and light intensity of 20 µmol m^{-2} s^{-1}, under 16 h photoperiod, until their use. For each plant type, about 100 plantlets were recovered from five glass jars (20 plantlets per each jar), in order to obtain a sufficient amount of vegetal material (0.5 – 1 g form each analysis) to be used for the extraction step. In addition, the collected material can be considered representative of the secondary metabolite content of the plantlets.

Sample extraction

Plantlets were firstly recovered from the culture medium and their root apparatus was gently washed with distilled water; after that, leaves and roots were excised and treated separately. Specifically, *vitro*-derived leaves and roots recovered from the different jars were weighted, lyophilized by a Freeze dryer Lio-5P (5Pascal, Milan, Italy), pulverized, and extracted with ethanol/water solution (80/20) with a dilution factor of 1:20, resorting to two extraction procedures: the first was based on the use of a shaker (HS 501 digital shaker, IKA-Werke GmbH & Co, Staufen, Germany; 200 strokes/minute for 2 h at room temperature), while for the second an ultrasound sonication bath (VWR International, Milan, Italy) was utilized, extracting samples for 30 min at 25 °C (Carbone et al. 2020; Martelli et al. 2020). The extracts were centrifuged at 5000 rpm for 10 min at room temperature (Centrifugette 4206 centrifuge, Alc International, Pévy, France) and the supernatants were diluted 1:5 with distilled water, for further analysis. Each extraction procedure was repeated twice. The moisture and the corresponding dry matter percentages of *vitro*-derived leaves and roots were calculated on the basis of lyophilisation procedure applied. All the results reported are expressed on the dry matter (DM).

Total phenolic content determination

The total phenolic content (TPC) was evaluated resorting to Folin–Ciocalteau's phenol reagent (Martelli et al. 2020), with some modifications. In brief, 250 µl of extract were mixed with 1 ml of an aqueous solution of Folin–Ciocalteau's phenol reagent (Sigma-Aldrich, St. Louis, MO, USA) (1/10 v/v), and 2 ml of an aqueous solution of sodium carbonate (20%, w/v), and kept in the dark for 30 min. The absorbance at 760 nm was measured using a spectrophotometer (JASCO V-530 spectrophotometer, Easton, MD, USA). To evaluate the quantity of polyphenols contained in the considered samples, a calibration curve was constructed, using gallic acid as reference in a concentration range of 10–100 mg/kg (5 points). All the analyses were repeated twice on each sample extract. In addition, in order to achieve more accurate results, the instrumental software was set up to perform three consecutive measurements on each analysed sample. The same approach was then applied for the other assays used to determine the antioxidant capacity of extracts. Concerning total polyphenolic content, results obtained for analysed samples were expressed as mg GAE/g DM.

Evaluation of antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant capacity of leaves and roots extracts was determined applying the DPPH radical scavenging assays (Abram et al. 2015), with slight modifications. 100 µl of sample extract or standard solution were added of 2.9 ml of a DPPH (Sigma-Aldrich, St. Louis, MO, USA) ethanolic solution (0.05 mM), and kept in the dark for 30 min. After that, the absorbance at 517 nm was measured (JASCO V-530 spectrophotometer, Easton, MD, USA). To evaluate the antioxidant capacity, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, St. Louis, MO, USA) was used as reference, preparing five different standard solutions (0.1–1 mM) that were utilized for the calibration curve construction. In addition, a blank constituted of 100 µl of extraction solution was also analysed under the same experimental conditions applied for samples. Antioxidant capacity was calculated on the basis of radical inhibition percentage (I%), as follows: I% = [(AbsB—AbsS)/AbsB]*100, were AbsB was the absorbance of the blank and AbsS was the absorbance of sample/Trolox standard solution. Results were then expressed as mg TEAC/ml (Trolox Equivalent Antioxidant Capacity). All the analyses were conducted in double, and three consecutive measurements were performed on each sample.

Determination of radical scavenging capacity by 2,2-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS) assay

To evaluate the radical scavenging activity of hop leaf and root extracts, a second test was conducted using as radical the ABTS (Wu et al. 2021). In particular, an aqueous ABTS (Sigma-Aldrich, St. Louis, MO, USA) solution (7 mM) containing $K_2O_8S_2$ (5 mM) was prepared and kept in the dark under stirring for 16 h. Then, the solution was diluted with bi-distilled water in order to obtain an $ABTS^{+\cdot}$ radical working solution with an absorbance of 0.7 ± 0.2 at 734 nm (JASCO V-530 spectrophotometer, Easton, MD, USA). For sample analyses, 1.9 ml of ABTS working solution was added at 100 µl of sample extract or standard solution, and the reaction was conducted in the dark, at room temperature for 30 min. A calibration curve

was prepared utilizing Trolox as reference, as described in the case of DPPH test. The absorbance values measured at 734 nm were used to calculate the I%, and to obtain the antioxidant activity expressed as mg TEAC/ml. All the analyses were repeated twice, and three consecutive measurements were performed on each sample.

Determination of ferric reducing power (FRAP)

The FRAP was determined as reported by Keskin et al. (2019). In brief, the working solution was prepared by mixing 25 ml of sodium acetate buffer solution (pH of 3.6 ± 0.1), 2.5 ml of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich, St. Louis, MO, USA), dissolved in 40 mM HCL, and 2.5 ml of an aqueous $FeCl_3 \cdot 6H_2O$ (20 mM), and warming this mixture at 37 °C for 30 min. After that, 150 µl of leaf and root extract or Trolox standard solution were mixed with 1.85 ml of the working solution, and the resulting mixture was kept in the dark at room temperature for 30 min. The absorbance of all the samples was measured at 593 nm (JASCO V-530 spectrophotometer, Easton, MD, USA), and the ferric reducing power was obtained on the basis of a Trolox calibration curve, in the same concentration range considered for the previous tests, and results were expressed as mg TEAC/ml. All the analyses were repeated twice, and three consecutive measurements were performed on each sample.

Qualitative characterization of extracts by UHPLC-MS/MS

Leaf and root extracts, fivefold diluted with 0.1% aqueous formic acid (Sigma-Aldrich, St. Louis, MO, USA), were used, also, to perform a full characterization. Analyses were performed through a UHPLC–MS/MS apparatus, composed of an UHPLC Ultimate 3000 separative module (Dionex, Sunnyvale, CA, USA), coupled with a TSQ Vantage triple quadrupole fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher, San Jose, CA, USA). The separation of compounds was performed using a RP-C_{18} SunShell column (2.6 µm, 100×2.10 mm; ChromaNik Technologies, Osaka, Japan) maintained at 40 °C. The mobile phase consisted in water (eluent A) and acetonitrile (eluent B), both acidified with 0.2% of formic acid (solvents and reagent from VWR, Milan, Italy). The elution gradient started at 1% of B for 1 min, then B % increased gradually at 80% after 13 min and it was maintained for 7 min for column washing. After that, the initial conditions were restored in 1 min, with a total run time of 22 min. The flow rate was set at 0.35 ml/min, and the injected sample volume was 2 µl.

For compound detection, MS experiments were performed in negative ionization mode. In detail, spray voltage was 3000 V, capillary temperature was set at 270 °C, while vaporizer temperature was 300 °C; nitrogen was used as both sheath and auxiliary gas, with a flow of 50 and 5 units, respectively. Sample analyses were initially carried out using a Full Scan method from m/z 100 to 1500, applying a scan time of 0.5 s. Then, target MS/MS analyses were carried out developing Product Ion Scan (PIS) experiments in order to obtain a better compound identification. Collision energy was set at 30 V and pure argon gas was used as collision gas. The identification of compounds listed in Table 4 was performed by comparing the MS/MS ion spectra with the fragmentation data available in several online libraries such as Mass Bank Europe, ReSpect, PubChem, and MoNA – Mass Bank of North America. Additional MS/MS information was obtained from previous works on hop-derived polyphenols, and other secondary metabolites (Česlová et al. 2009; Choi et al. 2018; Cirlini et al. 2016; Fernández-Poyatos et al. 2021; Helmja et al. 2007; McCallum et al. 2019; Prencipe et al. 2014; Xiang et al. 2021).

Statistical analysis

Data obtained from the chemical analyses of *vitro*-derived leaves and roots were subjected to three-way Analysis of Variance, considering the factors "Genotype", "Plant material" and "Extraction method"; mean separation has been carried out resorting to Tukey's test ($p \leq 0.05$) (SYSTAT 13).

Moreover, Pearson correlation test was performed to evaluate the relationship between the different antioxidant activity tests and the total polyohenolic content (IBM SPSS Statistics 26.0 software, SPSS Inc., Chicago, IL).

Results

Chemical characterization of *vitro*-derived root and leaf extracts from two hop genotypes, Cascade and Gianni

TPC and the antioxidant activity of all the different extracts are reported in Table 2. Leaves of both genotypes showed a total polyphenolic content (TPC) and antioxidant capacity higher than roots.

Statistical analysis evidenced that the only factor influencing the TPC parameter is, indeed, the "Plant Material" used to obtain the extract; in leaves a TPC significantly higher than roots was recorded (6.14 ± 2.26–8.00 ± 0.06 mg GAE/g SS vs. 3.92 ± 0.20–5.25 ± 0.44 mg GAE/g respectively), independently of the genotype or the extraction method applied.

Extracts obtained from *vitro*-derived leaves and roots of Gianni and Cascade, other than for TPC, were characterized in terms of antioxidant activity. To this aim, three different,

Table 2 Total phenolic content (TPC) and antioxidant activity, measured by DPPH, ABTS and FRAP assays, of extracts from hop *vitro*-derived leaves and roots of two genotypes, Gianni and Cascade (mean ± SD)

Genotype	Plant material	Extraction method	TPC		DPPH		ABTS		FRAP	
			mg GAE/g	± SD	mg TEAC/ml	± SD	mg TEAC/ml	± SD	mg TEAC/ml	± SD
Gianni	Roots	Ultrasound	4.33	1.51	5.59	1.90	19.10	1.15	9.76	3.12
		Shaker	3.92	0.04	6.56	0.85	20.75	0.62	9.22	1.18
	Leaves	Ultrasound	7.47	2.58	6.76	2.04	21.12	1.30	13.81	3.20
		Shaker	8.00	0.06	5.73	0.79	20.85	0.67	12.12	1.70
Cascade	Roots	Ultrasound	5.24	1.28	11.04	3.33	22.16	1.35	15.00	3.24
		Shaker	5.25	0.05	10.95	1.42	20.21	0.65	13.23	1.69
	Leaves	Ultrasound	6.14	2.26	8.56	3.24	20.76	1.24	9.37	3.89
		Shaker	7.94	0.75	8.72	1.07	20.55	3.17	11.81	1.37
Statistical analysis of factors[a]										
GENOTYPE (GEN)			0.705		**0.001**		0.499		0.280	
PLANT MATERIAL (PM)			**0.002**		0.166		0.694		0.981	
EXTRACTION METHOD (EM)			0.418		0.997		0.774		0.698	
GEN*EM			0.565		0.965		0.213		0.476	
GEN *PM			0.170		0.116		0.259		**0.007**	
PM*EM			0.340		0.559		0.949		0.452	
GEN*PM*EM			0.679		0.457		0.201		0.204	

[a]Three-way analysis of variance (ANOVA), Tukey's test ($p \leq 0.05$)

Authors put in bold the p values ≤0.05, indicating which factor was significant

Table 3 Results of two-tailed Pearson's correlation test for assessing relations between phenolic content and antioxidant capacity values

ASSAY		Gianni				Cascade			
		TPC	DPPH	FRAP	ABTS	TPC	DPPH	FRAP	ABTS
TPC	Pearson's correlation	1	0.434	0.867*	0.723	1	0.173	0.246	− 0.091
	Sign. (two-tailed)		0.389	**0.025**	0.105		0.743	0.638	0.865
DPPH	Pearson's correlation	0.434	1	0.708	0.792	0.173	1	0.914*	0.144
	Sign. (two-tailed)	0.389		0.115	0.06	0.743		**0.011**	0.786
FRAP	Pearson's correlation	0.867*	0.708	1	0.8	0.246	0.914*	1	0.174
	Sign. (two-tailed)	**0.025**	0.115		0.056	0.638	**0.011**		0.741
ABTS	Pearson's correlation	0.723	0.792	0.8	1	− 0.091	0.144	0.174	1
	Sign. (two-tailed)	0.105	0.06	0.056		0.865	0.786	0.741	

*The correlation is considered statistically significant at 0.05 (two-tailed)

but complementary, methods were applied: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-Azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS), and Ferric Reducing Antioxidant Power (FRAP) assays. Since spectrophotometric assays could not discriminate among compounds that present absorbance at similar wavelengths, thus lowering precision in testing the compounds of interest, it is advisable to perform more than one test based on electron or radical scavenging activity, such as DPPH, ABTS, ACA, or FRAP (Moon and Shibamoto 2009).

Differently from what observed for the TPC, the statistical analysis evidenced that "Genotype" is the only factor influencing the antioxidant capacity of the extracts (Table 2). Specifically, extracts recovered from Cascade samples, independently of the plant material and extraction method considered, have an antioxidant activity significantly higher than those obtained for Gianni extracts (9.23 mg TEAC/ml vs. 6.20 mg TEAC/ml) (Table 2).

Other than with DPPH assay, antioxidant activity of hop *vitro*-derived explants has been measured through ABTS assay; for this parameter, no significant differences were observed ($p > 0.05$; Table 2), nor on the basis of the genotype neither comparing the two starting plant materials used. These results probably depend on the composition of the antioxidant fraction, and on the different response that the compounds may have when subjected to reactions with different radicals.

In the case of results obtained for FRAP assay, a significant interaction between the factors "Plant Material" and "Genotype" was registered (Table 2); specifically, there is a significant difference, between Gianni and Cascade, in the antioxidant activity measured in roots. Indeed, antioxidant activity of extracts from Cascade roots is 1.5 fold higher than that of Gianni samples (14.11 mg TEAC/ml vs. 9.49 mg TEAC/ml).

The statistical analysis did not evidence significant influence exerted by the extraction method for any of the considered parameters; even though, for further analysis, the method based on the use of shaker was selected, because, observing the standard deviation, this method guarantees a better reproducibility (Table 2).

Correlation between phenolic concentration and antioxidant capacity was investigated by Pearson correlation test (Table 3) that evidenced, for Gianni, a significant correlation ($r = 0{,}867$), between the results of Folin–Ciocalteau test and those of FRAP assay, suggesting that the reducing activity is mainly due to phenolic compounds. Conversely, for Cascade, a significant correlation ($r = 0{,}914$) was registered between DPPH and FRAP assays, meaning that the antioxidant activity is exerted by the same compounds. Considering that the small molecules, acceding easier to the radical site of DPPH, are the most responsible of antioxidant activity, it is very likely that the reducing power, measured with FRAP assay, is due to the low molecular weight polyphenols.

Characterization of extracts from vitro-derived leaves and root of Cascade and Gianni, through UHPLC-MS/MS

Extracts from *vitro*-derived leaves and roots of Cascade and Gianni, after TPC and the antioxidant activity measurements, were further investigated on their qualitative profile, through UHPLC-MS/MS technique (Table 4).

The obtained profiles allowed the detection and the identification of 21 compounds in the extracts. Out of 21, only dihydroxybenzoic acid-*O*-hexoside and catechin were detected in all the analysed samples. Although Cascade extracts resulted richer in terms of number of compounds detected, the molecular profiles of the two analysed genotypes were almost similar. In addition, leaf extracts presented a more complex profile than that obtained from roots, where only few of the 21 identified compounds were present.

Table 4 UHPLC-MS/MS identification of polyphenolic compounds detected in extracts from hop leaves and roots (genotypes Cascade and Gianni)

Compound	RT	$[M-H]^-$ (m/z)	MS^2 ion fragments (m/z)	STD	Roots		Leaves		Reference
					Gianni	Cascade	Gianni	Cascade	
Dihydroxybenzoic acid-*O*-hexoside	3.79	315	153, 152, 109, 108		**x**	**x**	**x**	**x**	Pub Mass Bank Europe
Galloyl-*O*-hexoside	4.54	331	169, 125				**x**	**x**	Pub Mass Bank Europe
Catechin	5.62	289	245		**x**	**x**	**x**	**x**	Choi et al. (2018), ReSpect
Coumaroylquinic acid I	5.90	337	163, 119, 191					**x**	PubChem
Coumaroylquinic acid II	5.99	337	163, 119, 191					**x**	PubChem
3-Caffeoylquinic acid	5.90	353	191, 179, 135					**x**	Cirlini et al. (2016)
Sinapic acid-*O*-hexoside I	5.94	385	223, 208, 179, 164, 149, 121		**x**		**x**	**x**	Fernández-Poyatos et al. (2021)
5-Caffeoylquinic acid	6.08	353	191, 173, 135				**x**	**x**	Cirlini et al. (2016)
Sinapic acid-*O*-hexoside II	6.25	385	223, 208, 179, 164, 149, 121			**x**	**x**	**x**	Fernández-Poyatos et al. (2021)
Coumaroylquinic acid III	6.43	337	173, 191, 163, 119				**x**	**x**	Choi et al. (2018)
Coumaroylquinic acid IV	6.69	337	191, 173, 119					**x**	Choi et al. (2018)
Rutin (Quercetin-3-*O*-rutinoside)	6.72	609	300,301,255	x			**x**	**x**	ReSpect Standard compound
Kaempferol-*O*-rutinoside I	6.79	593	447, 389, 285, 284, 255				**x**	**x**	McCallum et al. (2019)
Kaempferol-*O*-rutinoside II	7.00	593	357, 285, 284, 257, 231					**x**	Pub Mass Bank Europe
Isoxanthohumol	9.22	353	119	x				**x**	Helmja et al. (2007) Standard compound
Xanthohumol	11.42	353	295, 189, 133, 119	x			**x**	**x**	Standard compound
Lupulone	12.09	413	301,289,369	x	**x**	**x**		**x**	Standard compound
Cohumulone	14.13	347	278, 235, 223, 209, 207, 195, 194, 193				**x**	**x**	, MoNA – Mass Bank of North America; Pub Mass Bank Europe
Post-lupulone	14.53	385	273					**x**	Helmja et al. (2007)
Humulone/Adhumulone	14.73	361	292,249				**x**	**x**	Česlová et al. (2009), Helmja et al. (2007), and Prencipe et al. (2014)
Colupulone	15.46	399	287,275,219,207			**x**		**x**	Helmja et al. (2007), ReSpect

Discussion

The use of plant biomass as bioactive compound source is biased by the susceptibility of secondary metabolism to the growing plant conditions. *Vitro*-derived plantlets, growing in a controlled environment and in standardized conditions, represent an invaluable matrix for a constant and continuous bioactive compound production. In this study, to evaluate the total polyphenolic content and the antioxidant activity of extracts obtained from *vitro*-derived hop plantlets, and to set up a valid extraction protocol, two hop genotypes, two types of starting material and two extraction procedures were investigated. Results regarding the TPC parameter evidenced that *vitro*-derived hop leaves are richer in polyphenolic compounds than roots. Due to the lack of literature on chemical characterization of *vitro*-derived hop plantlets, results reported in this study were compared with those described for cones, leaves, and roots collected from in field grown plants of hop and/or of related species. As an example, Choi et al. (2018) described the highest polyphenol concentration obtained in leaves, rather than in roots or stems of Korean *Humulus japonicus*. TPC content could be influenced by several factors, among which starting material, extraction method, solvent treatments, and genotype (Abram et al. 2015). *Vitro*-derived plantlets used in the present study had a lower TPC than cones of Magnum and Marynka (Kovwalczyk et al. 2013), but higher than leaves collected from field, as reported by Proestos et al. (2006).

TPC recorded in this study seems to be independent of the adopted extraction procedure; in literature, TPC lower than that of *vitro*-derived leaves was obtained resorting to the same extraction solvent (70% ethanol) (Muzykiewicz et al. 2019), while, when methanol was used, results comparable with those reported in the current study were obtained (Keskin et al. 2019). Another important factor influencing the TPC content is the genotype of starting material (Abram et al. 2015; Čeh et al. 2007), differently, the two genotypes analysed in this study presented comparable TPC content in their leaves.

The information given by the parameter TPC was completed carrying assays aimed at determining the extracts antioxidant activity. Results obtained with the DPPH assay revealed that extracts from Cascade evidenced an antioxidant capacity higher than those from Gianni. Moreover, DPPH antioxidant activity described in this study is higher than values reported on extracts obtained from open field hop leaves (Abram et al. 2015; Muzykiewicz et al. 2019). Finally, DPPH values reported in this study are comparable, sometimes lower (Thiruvengadam et al. 2015), sometimes higher (Amoo et al. 2012) than those reported in other *vitro*-derived matrices. So, the results on antioxidant activity support the thesis of possible use of *vitro*-derived hop material as antioxidant source, after a genotype characterization. Different assays may give different responses, even considering the same starting material and extraction procedure; this is what was observed evaluating the results of ABTS assay that did not evidence any difference for any factor analysed, in contrast with information reported by Choi et al. (2018) who evidenced a radical scavenging capacity higher in hop leaves than in roots. These results probably depend on the composition of the polyphenolic fraction, and on the different response that these compounds may have when subjected to reactions with different radicals. Regarding FRAP assay, the two genotypes responded differently. Cascade root extract antioxidant activity was two-fold higher than that of Gianni; similar trend was reported for cultivars Aurora and H. Magnum, for which a differential genotype response was obtained according to the starting material considered, leaves or cones (Abram et al. 2015). Furthermore, in this study, the factor "Starting material" alone did not seem to exert the same impact, while Choi et al. (2018) report that leaves of *H. japonicus* showed a reducing power, measured by FRAP method, higher than that of roots and stems.

Regarding the molecular profile obtained by UHPLC-MS/MS technique, the identified compounds (Table 4) belong to several classes, xanthohumol (prenylated chalcone), isoxanthohumol (prenylated flavanone), kaempferol-3-rutinoside and rutin (glycosylated flavonols), catechin (flavan-3-ols) and phenolic acids, derived both from benzoic acid and cinnamic acid. Of particular interest is the presence of phloroglucinol derivatives, a class of compounds that particularly characterize the hop plant. In detail, α-acids, such as humulone/adhumulone and co-humulone, and β-acids, such as lupulone, colupulone and post-lupulone were the most characteristic components, especially in Cascade leaf extracts. These compounds, typically present in hop cones (Česlová et al. 2009; Helmja et al. 2007; Magalhães et al. 2010; Santagostini et al. 2020) gained valuable interest because of their bioactive properties. Lupulones and humulones are known, indeed, for their antibacterial and antifungal activity, being inhibitor of diacylglycerol acetyltransferase, limiting lipid metabolism, and as a consequence, affecting the composition of cell wall and microorganism membrane (Bocquet et al. 2018), while α-acids and β-acids, and xanthohumol resulted active components against *Listeria monocytogenes* and *Staphylococcus aureus* (Kramer et al. 2015).

Other than the most characteristic hop compounds, also flavan-3-ol and flavonol glycosides, as catechin, rutin and kaempferol-*O*-rutinoside, have been identified in hop cones by Kavalier et al. (2011), and rutin have been detected in strobiles by Magalhães et al. (2010).

Among all identified polyphenolics, few molecules have already been characterized in hop tissues different from cones, such as leaves, roots and stems, as caffeolquinic acids, coumaroylquinic acids, and catechin. These compounds have been, previously, reported in the LC–MS/MS profile obtained from hop tissue extracts, prepared with ethyl acetate (Choi et al. 2018). Differences between the results obtained in the present study and those described by literature could depend on the different investigated plant material (in vitro versus in field plants), as from other parameters, as the solvent type used for the extraction. It was demonstrated that using different solvent mixtures, the profile of secondary metabolites changes, both in number of recovered components as in terms of compound concentration. Helmja et al., in 2007, investigated, by HPLC–MS/MS technique, the composition of hop strobilus extracts, obtaining using different solvent mixtures (methanol, water, acetone, etc.), and different extraction techniques, that polyphenolic and bitter acid profile depended on the method used to recover these metabolites. Differences can be pointed out also considering diverse plant materials and varieties. The present study highlighted, indeed, different profiles for leaves and roots, as between the two plant types, showing that Cascade deriving extracts presented a larger pattern of compounds of potential interest (Table 4). The wider range of polyphenols was indeed observed in Cascade leaves extracts: coumaroylquinic acids, 3-caffeoylquinic acid, and kaempferol-O-rutinoside I and II were not detected in Gianni extracts, while profiles obtained for roots resulted, in general, scarcer. Similar observations can be made regarding isoxanthohumol, and β-acids, as lupulone and colupulone, found in Cascade rather than in Gianni leaves, as more in general for all the α- and β-acids identified, contained prevalently in

leaves. The presence and concentration of xantohumol and α-acids is, actually, related to hop variety, as demonstrated by Mongelli et al., in 2015. The information about secondary metabolite composition could be useful in the choice of the more promising plant types suitable to give extracts abundant in bioactive components.

Conclusions

In the recent years, great interest has aroused about the potential use of bioactive compounds of natural origin, both from food and non-food enterprises. Other than from in field grown plants, it has been demonstrated that these compounds can be extracted from *vitro*-derived plant material, with the enormous advantage of guaranteeing a continuous and standardized biomass production. Hop (*Humulus lupulus* L.) plant, in all its parts, is a precious source of bioactive compounds, but its in vitro grown plant material has never been investigated; with this aim, the present study has been carried out to characterize leaves and roots of two hop plant types, Cascade and Gianni, cultivated in vitro.

In conclusion, between the two genotypes considered, explants from the variety Cascade seem to be the most promising, both in terms of total phenolic content and polyphenol profile, but the ecotype Gianni could be considered as interesting as other commercial hop varieties. Extracts obtained from both plant types turned out to contain hop characteristic compounds, such as α-acids and β-acids, as well as xanthohumol, that have been related to antioxidant, anti-inflammatory, estrogenic, sedative and antimicrobial activities. These results confirm the valuable potential of *vitro*-derived plant material as valid source of bioactive compounds, to be exploit in several industrial sectors, in a constant and sustainable manner, independently of the environment conditions and the season. To sum up, this study led new insights for further research, aimed at increasing and diversifying bioactive compound synthesis, as well as, at widening the number of hop genotypes chemically characterised.

Funding Open access funding provided by Università degli Studi di Parma within the CRUI-CARE Agreement. This research was supported by the project: "HOPLURE—Hop Leaves, an Unexplored REsource" (D91B21005370003), funded by University of Parma, through the action Bando di Ateneo 2021 per la ricercar, and co-funded by MUR-Italian Ministry of Universities and Research—D.M. 737/2021—PNR—PNRR – NextGenerationEU.

Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

References

Abram V, Čeh B, Vidmar M, Hercezi M, Lazić N, Bucik V, Smole Možina S, Košir IJ, Kač M, Demšar L, Poklar Ulrih N (2015) A comparison of antioxidant and antimicrobial activity between hop leaves and hop cones. Ind Crops Prod. https://doi.org/10.1016/j.indcrop.2014.11.008

Alonso-Esteban JI, Pinela J, Barros L, Ciri´c A, Sokovi´c M, Calhelha RC, Torija-Isasa E, de Cortes ´Sánchez-Mata M, Ferreira ICFR (2019) Phenolic composition and antioxidant, antimicrobial and cytotoxic properties of hop (*Humulus lupulus* L.) seeds. Ind Crops Prod 134:154–159. https://doi.org/10.1016/j.indcrop.2019.04.001

Amoo SO, Aremu AO, Van Staden J (2012) *In vitro* plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. Plant Cell Tissue Organ Cult 111:345–358. https://doi.org/10.1007/s11240-012-0200-3

Anand S (2010) Various approaches for secondary metabolite production through plant tissue culture. Pharmacia 1:1–7

Astray G, Gullón P, Gullón B, Munekata PES, Lorenzo JM (2020) *Humulus lupulus* L. as a natural source of functional biomolecules. Appl Sci 10:5074. https://doi.org/10.3390/app10155074.

Benelli R, Vene` R, Ciarlo M, Carlone S, Barbieri O, Ferrari N (2012) The AKT/NF-jB inhibitor xanthohumol is a potent antilymphocytic leukemia drug overcoming chemoresistance and cell infiltration. Biochem Pharmacol 83:1634–1642. https://doi.org/10.1016/j.bcp.2012.03.006

Bhattacharya S, Virani S, Zavro M, Haas GJ (2003) Inhibition of Streptococcus mutans and Other Oral streptococci by hop (*Humulus lupulus* L.) constituents. Econ Bot 57:118–125. https://doi.org/10.2307/4256647

Bocquet L, Rivière C, Dermont C, Samaillie J, Hilbert JL, Halama P, Siah A, Sahpaz S (2018) Antifungal activity of hop extracts and compounds against the wheat pathogen *Zymoseptoria tritici*. Ind Crops Prod 122:290–297. https://doi.org/10.1016/j.indcrop.2018.05.061

Buchanan BB, Jones RL (2000) Biochemistry & molecular biology of plants. American Society of Plant Physiologists, Rockville. https://doi.org/10.1023/A:1013849028622.

Carbone K, Macchioni V, Petrella G, Cicero DO (2020) Exploring the potential of microwaves and ultrasounds in the green extraction of bioactive compounds from *Humulus lupulus* for the food and pharmaceutical industry. Ind Crops Prod 156:112888. https://doi.org/10.1016/j.indcrop.2020.112888

Čeh B, Kac M, Košir IJ, Abram V (2007) Relationships between xanthohumol and polyphenol content in hop leaves and hop cones

with regard to water supply and cultivar. Int J Mol Sci 8(9):989–1000. https://doi.org/10.3390/i8090989
Češlová L, Holčapek M, Fidler M, Drštičková J, Lísa M (2009) Characterization of prenylflavonoids and hop bitter acids in various classes of Czech beers and hop extracts using high-performance liquid chromatography–mass spectrometry. J Chromatogr A 1216:7249–7257. https://doi.org/10.1016/j.chroma.2009.09.022
Chadwick LR, Pauli GF, Farnsworth NR (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. Phytomedicine 13:119–131. https://doi.org/10.1016/j.phymed.2004.07.006
Choi JY, Desta KT, Lee SJ, Kim YH, Shin SC, Kim GS, Lee SJ, Shim JH, Jae H, Hacımüftüo A, Abd El-Aty AM (2018) LC-MS/MS profiling of polyphenol-enriched leaf, stem and root extracts of Korean *Humulus japonicus* Siebold & Zucc and determination of their antioxidant effects. Biomed Chromatogr 32:1–8. https://doi.org/10.1002/bmc.4171
Cirlini M, Mena P, Tassotti M, Herrlinger KA, Nieman KM, Dall'Asta C, Del Rio D (2016) Phenolic and volatile composition of a dry spearmint (*Mentha spicata* L.) extract. Molecules 21(8):1007. https://doi.org/10.3390/molecules21081007
Dal Toso R, Melandri F (2011) Sustainable sourcing of natural food ingredients by plant cell cultures. Agro Food Ind Hi Tech 22:26–28
Delmulle L, Bellahcène A, Dhooge W, Comhaire F, Roelens F, Huvaere K, Heyerick A, Castronovo V, De Keukeleire D (2006) Anti-proliferative properties of prenylated flavonoids from hops (*Humulus lupulus* L.) in human prostate cancer cell lines. Phytomedicine 13:732–734. https://doi.org/10.1016/j.phymed.2006.01.001
Dias MI, Sousa MJ, Alves RC (2016) Ferreira ICFR (2016) Exploring plant tissue culture to improve the production of phenolic compounds: a review. Ind Crops Prod 82:9–22. https://doi.org/10.1016/j.indcrop.2015.12.016
FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. Low cost options for tissue culture technology in developing countries. In: Proceedings of a Technical Meeting, Viena. 2002.
Fernández-Poyatos MDP, Llorent-Martínez EJ, Ruiz-Medina A (2021) Effect of ripening on the phenolic composition and mineral content of three varieties of olive fruits. Foods 10(2):380. https://doi.org/10.3390/foods10020380
Gerhäuser C (2005) Broad spectrum anti-infective potential of xanthohumol from hop (*Humulus lupulus* L.) in comparison with activities of other hop constituents and xanthohumol metabolites. Mol Nutr Food Res 49(9):827–831. https://doi.org/10.1002/mnfr.200500091
Green PB (1997) Self-organization and the formation of patterns in plants. In: Alt W, Deutsch A, Dunn GA (eds) Dynamics of cell and tissue motion. mathematics and biosciences in interaction. Birkhäuser, Basel. pp 243–249.
Haas GJ, Barsoumian R (1994) Antimicrobial activity of hop resins. Food Prot 57:59–61. https://doi.org/10.4315/0362-028X-57.1.59
Hall AJ, Babish JG, Darland GK, Carroll BJ, Konda VR, Lerman RH, Bland JS, Tripp ML (2008) Safety, efficacy and anti-inflammatory activity of rho iso-alpha-acids from hops. Phytochemistry 69(7):1534–1547. https://doi.org/10.1016/j.phytochem.2008.02.001
Helmja K, Vaher M, Püssa T, Kamsol K, Orav A, Kaljurand M (2007) Bioactive components of the hop strobilus: comparison of different extraction methods by capillary electrophoretic and chromatographic methods. J Chromatogr A 1155:222–229. https://doi.org/10.1016/j.chroma.2006.12.067
Hrnčič MK, Španinger E, Košir IJ, Knez Ž, Bren U (2019) Hop compounds: extraction techniques, chemical analyses, antioxidative, antimicrobial, and anticarcinogenic effects. Nutrients 11:257. https://doi.org/10.3390/nu11020257
Karabín M, Hudcová T, Jelínek L, Dostálek P (2016) Biologically active compounds from hops and prospects for their use. Compr Rev Food Sci Food Saf 15:542–567. https://doi.org/10.1111/1541-4337.12201
Kavalier AR, Litt A, Ma C, Pitra NJ, Coles MC, Kennelly EJ, Matthews PD (2011) Phytochemical and morphological characterization of hop (*Humulus lupulus* L.) cones over five developmental stages using high performance liquid chromatography coupled to time-of-flight mass spectrometry, ultrahigh performance liquid chromatography photodiode array detection, and light microscopy techniques. J Agric Food Chem 59:4783–4793. https://doi.org/10.1021/jf1049084
Keskin Ş, Şirin Y, Çakir HE, Keskin M (2019) An investigation of *Humulus lupulus* L.: phenolic composition, antioxidant capacity and inhibition properties of clinically important enzymes. S Afr J Bot 120:170–174. https://doi.org/10.1016/j.sajb.2018.04.017
Kitaoka M, Kadokawa H, Sugano M, Ichikawa K, Taki M, Takaishi S, Iijima Y, Tsutsumi S, Boriboon M, Akiyama T (1998) Prenylflavonoids: a new class of non-steroidal phytoestrogen (Part 1). Isolation of 8-isopentenylnaringenin and an initial study on its structure–activity relationship. Planta Med 64:511–515. https://doi.org/10.1055/s-2006-957504
Kovwalczyk D, Świeca M, Cichocka J, Gawlik-Dziki U (2013) The phenolic content and antioxidant activity of the aqueous and hydroalcoholic extracts of hops and their pellets. J Inst Brew 119:103–110. https://doi.org/10.1002/jib.73
Kramer B, Thielmann J, Hickisch A, Muranyi P, Wunderlich J, Hauser C (2015) Antimicrobial activity of hop extracts against foodborne pathogens for meat applications. J Appl Microbiol 118:648–657. https://doi.org/10.1111/jam.12717
Krottenthaler M (2009) Hops. In: Eßlinger HM (ed) Handbook of brewing: processes, technology, markets. Wiley, Weinheim, pp 85–104
Lee Y-M, Hsieh K-H, Lu W-J, Chou H-C, Chou D-S, Lien L-M, Sheu J-R, Lin K-H (2012) Xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus*), prevents platelet activation in human platelets. Evid-Based Complement Altern Med. https://doi.org/10.1155/2012/852362
Liu M, Hansen P, Wang G, Qui L, Dong J, Yin H, Zhonghua Q, Mei Y, Jinlai M (2015) Pharmacological profile of xanthohumol a prenylated flavonoid from hops (*H. lupulus*). Molecules 20:754–779. https://doi.org/10.3390/molecules20010754
Magalhães PJ, Vieira JS, Gonçalves LM, Pacheco JG, Guido LF, Barros AA (2010) Isolation of phenolic compounds from hop extracts using polyvinylpolypyrrolidone: characterization by high-performance liquid chromatography–diode array detection–electrospray tandem mass spectrometry. J Chromatogr A 1217:3258–3268. https://doi.org/10.1016/j.chroma.2009.10.068
Martelli F, Cirlini M, Lazzi C, Neviani E, Bernini V (2020) Edible seaweeds and spirulina extracts for food application: in vitro and in situ evaluation of antimicrobial activity towards foodborne pathogenic bacteria. Foods 9(10):1442. https://doi.org/10.3390/foods9101442
Mass Bank Europe. https://massbank.eu/MassBank/.
McCallum JL, Nabuurs MH, Gallant ST, Kirby CW, Mills AAS (2019) Phytochemical characterization of wild hops (*Humulus lupulus* ssp. lupuloides) germplasm resources from the Maritimes region of Canada. Front Plant Sci 10:1438. https://doi.org/10.3389/fpls.2019.01438
Milligan SR, Kalita JC, Heyerick A, Rong H, De Cooman L, De Keukeleire D (1999) Identification of a potent phytoestrogen in hops (*Humulus lupulus* L.) and beer. J Clin Endocrinol Metab 84:2249–2252. https://doi.org/10.1210/jcem.84.6.5887
Milligan SR, Kalita JC, Pocock V, Van De Kauter V, Stevens JF, Deinzer ML, Rong H, De Keukeleire D (2000) The endocrine activities of 8-prenylnaringenin and related hop (*Humulus lupulus*

L.) flavonoids. J Clin Endocrinol Metab 85:4912–4915. https://doi.org/10.1210/jcem.85.12.7168
MoNA – Mass Bank of North America. https://mona.fiehnlab.ucdavis.edu/.
Mongelli A, Rodolfi M, Ganino T, Marieschi M, Dall'Asta C, Bruni R (2015) Italian hop germplasm: characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis. Ind Crops Prod 70:16–27. https://doi.org/10.1016/j.indcrop.2015.02.036
Moon JK, Shibamoto T (2009) Antioxidant assays for plant and food components. J Agric Food Chem 57(5):1655–1666. https://doi.org/10.1021/jf803537k
Murashige T, Skoog FA (1962) Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15:473–479. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
Muzykiewicz A, Nowak A, Zielonka-Brzezicka J, Florkowska K, Duchnik W, Klimowicz A (2019) Comparison of antioxidant activity of extracts of hop leaves harvested in different years. Herba Polonica 65(3):1–9. https://doi.org/10.2478/hepo-2019-0013
Nezi P, Cicaloni V, Tinti L, Salvini L, Iannone M, Vitalini S, Garzoli S (2022) Metabolomic and proteomic profile of dried hop inflorescences (*Humulus lupulus* L. cv. Chinook and cv. Cascade) by SPME-GC-MS and UPLC-MS-MS. Separations 9, 204. https://doi.org/10.3390/separations9080204.
Nionelli L, Pontonio E, Gobbetti M, Rizzello CG (2018) Use of hop extract as antifungal ingredient for bread making and selection of autochthonous resistant starters for sourdough fermentation. Int J Food Microbiol 266:173–182. https://doi.org/10.1016/j.ijfoodmicro.2017.12.002
Nuutinen T (2018) Medicinal properties of terpenes found in Cannabis sativa and Humulus lupulus. Eur J Med Chem 157:198–228. https://doi.org/10.1016/j.ejmech.2018.07.076
Prencipe FP, Brighenti V, Rodolfi M, Mongelli A, Dall'Asta C, Ganino T, Bruni R, Pellati F (2014) Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in *Humulus lupulus* L. J Chromatogr A 1349:50–59. https://doi.org/10.1016/j.chroma.2014.04.097
Proestos C, Boziaris IS, Nychas GJE, Komaitis M (2006) Analysis of flavonoids and phenolic acid in Greek aromatic plants: investigation of their antioxidant capacity and antimicrobial activity. Food Chem 95(4):664–671. https://doi.org/10.1016/j.foodchem.2005.01.049
PubChem. https://pubchem.ncbi.nlm.nih.gov/.
Rao RS, Ravishankar GA (2002) Plant tissue cultures; chemical factories of secondary metabolites. Biotechnol Adv 20:101–153. https://doi.org/10.1016/s0734-9750(02)00007-1
ReSpect. http://spectra.psc.riken.jp/menta.cgi/index
Rodolfi M, Chiancone B, Liberatore CM, Fabbri A, Cirlini M, Ganino T (2019) Changes in chemical profile of Cascade hop cones according to the growing area. J Sci Food Agric 99:6011–6019. https://doi.org/10.1002/jsfa.9876
Roehrer S, Stork V, Ludwig C, Minceva M, Behr J (2019) Analyzing bioactive effects of the minor hop compound xanthohumol C on human breast cancer cells using quantitative proteomics. PLoS ONE 14:e0213469. https://doi.org/10.1371/journal.pone.0213469
Santagostini L, Caporali E, Giuliani C, Bottoni M, Ascrizzi R, Araneo SR, Papini A, Flamini G, Fico G (2020) Humulus lupulus L. cv. Cascade grown in Northern Italy: morphological and phytochemical characterization. Plant Biosys 154(3):316–325. https://doi.org/10.1080/11263504.2019.1610111
Sanz V, Torres MD, López Vilariño JM, Domínguez H (2019) What is new on the hop extraction? Trends Food Sci Technol 93:12–22. https://doi.org/10.1016/j.tifs.2019.08.018
Scarpa GM, Prota V, Schianchi N, Manunta F (2022) In vitro cultures for the production of secondary metabolites. In: Vijayakumar R, Raja SSS (eds) Secondary metabolites—trends and reviews. IntechOpen, London
Siragusa GR, Haas GJ, Matthews PD, Smith RJ, Buhr J, Dale NM, Wise MG (2008) Antimicrobial activity of lupulone against Clostridium perfrigens in the chicken intestinal tract jejunum and caecum. J Antimicrobial Chemotherapt 61:853–858. https://doi.org/10.1093/jac/dkn024
Srinivasan V, Goldberg D, Haas GJ (2004) Contribution to the antimicrobial spectrum of hop constituents. Economic Bot 58:230–238. https://doi.org/10.2307/4256920
Stevens JF, Ivancic M, Hsu VL, Deinzer ML (1997) Prenylflavonoids from Humulus lupulus. Phytochem 44(8):1575–1585. https://doi.org/10.1016/S0031-9422(96)00744-3
Tabata N, Ito M, Tomoda H, Omura S (1997) Xanthohumols, diacylglycerol acyltransferase inhibitors, from Humulus lupulus. Phytochemistry 46:683–687. https://doi.org/10.1016/S0031-9422(97)00157-X
Tapiero H, Tew KD, Ba GN, Mathe´ G (2002) Polyphenols: do they play a role in the prevention of human pathologies? Biomed Pharmacother 56:200–207. https://doi.org/10.1016/S0753-3322(02)00178-6
Thiruvengadam M, Gurunathan S, Chung IM (2015) Physiological, metabolic, and transcriptional effects of biologically-synthesized silver nanoparticles in turnip (*Brassica rapa* ssp. *rapa* L.). Protoplasma 252(4):1031–1046. https://doi.org/10.1016/j.tifs.2019.08.018
Tyrrell E, Archer R, Skinner GA, Singh K, Colston K, Driver C (2010) Structure elucidation and an investigation into the *in vitro* effects of hop acids on human cancer cells. Phytochem Lett 3(1):17–23. https://doi.org/10.1016/j.phytol.2009.10.006
Tyrrell E, Archer R, Tucknott M, Colston K, Pirianov G, Ramanthan D, Dhillon R, Siclair A, Skinner GA (2012) The synthesis and anticancer effects of a range of natural and unnatural hop β-acids on breast cancer cells. Phytochem Lett 5(1):144–149. https://doi.org/10.1016/j.phytol.2011.11.011
Van Cleemput M, Cattoor K, De Bosscher K, Haegeman G, De Keukeleire D, Heyerick A (2009) Hop (*Humulus lupulus*)-derived bitter acids as multipotent bioactive compounds. J Nat Prod 72:1220–1230. https://doi.org/10.1021/np800740m
Wu C-N, Sun L-C, Chu Y-L, Yu R-C, Hsieh C-W, Hsu H-Y, Hsu F-C, Cheng K-C (2020) Bioactive compounds with anti-oxidative and anti-inflammatory activities of hop extracts. Food Chem 330:127244. https://doi.org/10.1016/j.foodchem.2020.127244.
Wu L, Zhou K, Chen F, Chen G, Yu Y, Lv X, Zhang Wen, Rao P, Ni L (2021) Comparative study on the antioxidant activity of monascus yellow pigments from two different types of hongqu—functional Qu and coloring Qu. Front Microbiol. https://doi.org/10.3389/fmicb.2021.71529.
Xiang Z, Lin C, Zhu Y, Deng J, Xia C, Chen J (2021) Phytochemical profiling of antioxidative polyphenols and anthocyanins in the wild plant *Campanumoea lancifolia* (Roxb.) Merr. Int J Food Prop 24(1):105–114. https://doi.org/10.1080/10942912.2020.1867570.
Yong WK, Ho YF, Malek SN (2015) Xanthohumol induces apoptosis and S phase cell cycle arrest in A549 non-small cell lung cancer cells. Pharmacogn Mag 11(2):275–283. https://doi.org/10.4103/0973-1296.166069.
Zanoli P, Zavatti M (2008) Pharmacognostic and pharmacological profile of *Humulus lupulus* L. J Ethnopharmacol 116:383–396. https://doi.org/10.1016/j.jep.2008.01.011