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Volatile and Non-Volatile Content Determination and Biological Activity Evaluation of Fresh *Humulus lupulus* L. (cv. Chinook) Leaves and Inflorescences

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Abstract: In this work, the fresh leaves and inflorescences of *Humulus lupulus* L. cv. Chinook hops were investigated in order to describe their chemical composition and evaluate their biological activities. The analyses were carried out first on fresh untreated samples and then on pulverized ones using the SPME-GC-MS technique. In total, forty-two molecules belonging to different chemical classes were identified, and among these, twenty-three were terpene compounds. In order to carry out the activity assays, the powders were subjected to extraction with two different solvents (methanol and distilled water) by stirring and subsequent sonication at room temperature. To chemically characterize the extracts, the methanolic ones were analyzed by direct injection into the GC-MS apparatus, while the aqueous ones were analyzed by DI-SPME-GC-MS. In addition, with the aim to obtain information on the non-volatile content of the methanolic extracts, they were also subjected to derivatization, and the silylated derivatives were analyzed by GC-MS. The antioxidant activity was then evaluated by means of DPPH and ABTS assays after the determination of the total content of polyphenols and flavonoids. The greatest effects were observed on the methanolic extracts rather than on the aqueous ones. Furthermore, a preliminary study on the cytotoxic power of the methanolic extracts was also conducted on three different human cancer cell lines, such as non-small cell lung cancer (H1299), melanoma (A375) and breast cancer (MCF7). The obtained results showed that the two extracts induced a marginal effect on reducing breast tumor, melanoma and non-small cell lung cancer cell proliferation.

Keywords: volatile and non-volatile organic compounds; DI-SPME-GC-MS analysis; silylation; hops extract; antiradical activity; anticancer activity

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1. Introduction

Humulus lupulus L. cv. Chinook is the result of a cross between Petham Golding (female) and a wild American hops (male) developed under the USDA hops breeding program with a long process of research and selection, released to the brewing public and commercialized in 1985 [1]. It is characterized by a very high yield, an excellent adaptation to warm continental climates and a rather late ripening of the cones. Chinook has a high value in alpha acids and an unmistakable profile with an intense aroma of citrus fruits, especially grapefruit, resinous and spicy [2]. Its extreme versatility has led brewers to use Chinook in many styles, from IPA (Indian Pale Ales) to APA (American Pale Ales), stouts and barley wines [3].

In general, *H. lupulus* is a member of a small genus of flowering plants belonging to the Cannabaceae family, native to the temperate northern hemisphere (Europe, southwestern Asia, and North America) [4]. Nevertheless, it is now more widely cultivated (e.g., in Australia, New Zealand and South Africa) and used in brewing industries around the world with an annual production ranging between 80,000 and 100,000 tons and a value of approximately EUR 700 million [5,6].

The use of different hops parts (inflorescences, young branches and shoots) in folk medicine, in traditional cooking and in other fields is well-known [7], and its phytochemistry is extensively documented [4]. Hops contain hundreds of chemical compounds, including some secondary metabolites (polyphenols, resins, terpenes), with potential pharmacological and medicinal value that deserve to be explored [8,9].

New hops cultivars are currently being developed and tested. Chinook is one of several hundred cultivars already grown, and it is very common in Italian hops fields. The recent expansion of the craft beer market in Italy corresponds to a positive trend in the presence of dedicated farms, which has contributed to the rediscovery of this crop. Female inflorescences are mainly used in the beer industry, and almost all hops production is destined for microbreweries. Despite this, the hops supply chain, from the point of view of a circular economy of agri-food waste, is interesting. Several companies have also successfully experimented with hops in the food and cosmetics sector by exploiting its by-products, including leaves, green shoots and stems.

In our previous studies, the chemical profile of the essential oil and hydrolate of Chinook hops was characterized, and the corresponding apoptotic activity, as well as the metabolic and proteomic profile of the dried inflorescences, were investigated [3,10].

This work is a part of a larger project on hops and related products aimed at identifying active ingredients useful for the various fields of the scientific sector. Attention was therefore focused both on the leaves and on the inflorescences (Figure 1A,B). For the first time, the volatile and non-volatile content of fresh samples grown in peculiar pedoclimatic conditions, was determined using different methodologies. Their effects as antioxidant and cytotoxic agents were also evaluated.

(**A**) (**B**)

Figure 1. *H. lupulus* cv. Chinook fresh leaves (**A**) and inflorescences (**B**).

2. Materials and Methods

2.1. Plant Material

The Chinook hops investigated in this study, were provided by the certified organic farm "Alpe di Puntato" located in the Municipality of Stazzema (LU, Italy) at 1150 m a.s.l., immersed in the Park of the Apuan Alps of upper Versilia, more precisely in the locality "Paravii (44°04′59.416″; 10°29′49.408″). They were grown within the "Torbia di Fociomboli", called "Padule".

Usually, hops grow at altitudes below 800 m, but a series of favorable factors, such as the optimal exposure of the valley and the particularly fertile soil thanks to the spring water of the Apuan Alps used for the irrigation of Chinook hops through special tanks,

allowed a good harvest even in the first year of experimental cultivation. The inflorescences were harvested in September. The yield was around 300 g of fresh hops per plant, with high lupulin content cones. Afterwards, the fresh hops were vacuum packed until use (unpowdered sample). The collection was carried out in September 2022.

2.2. Materials

Ethanol and methanol solvents, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt), Folin & Ciocalteu's phenol reagent, $K_2S_2O_8$ (potassium persulfate), sodium carbonate, aluminum chloride, gallic acid, potassium acetate and quercetin were purchased from Merck (Darmstadt, Germany).

DMSO (dimethyl sulfoxide) and MTT (3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma Aldrich (St. Louis, MO, USA). For extraction and derivatization, acetone, pyridine and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.3. Extraction Process

Fresh inflorescences and leaves of Chinook hops were extracted by the same procedure [11] with two different solvents, namely distilled water and methanol, obtaining four extracts indicated respectively as IAE (inflorescence aqueous extract), LAE (leaf aqueous extract), IME (inflorescence methanolic extract) and LME (leaf methanolic extract). Both plant parts were manually pulverized in liquid nitrogen (powdered samples). Then, they were transferred to a flask, where MeOH or distilled water (3 mL/g) was added. Each mixture was sonicated for 10 min and subsequently stirred for 20 min. After a settling period, the supernatants were collected, placed in Eppendorf tubes and centrifuged (10,000× g, 10 min). The process was repeated twice, and the obtained extracts were combined after being filtered (0.22 µm pore size). The solvents were completely removed under reduced pressure using a rotary evaporator (RV 08-VC, IKA, Staufen, Germany), and the dry residues were stored until use. Suitably re-dissolved in water, methanol or water:methanol (1:1), they were tested and chemically analysed.

2.4. SPME Sampling

Thanks to application of the solid phase microextraction (SPME) sampling technique, the volatile chemical composition of the fresh and powder of inflorescences and leaves of Chinook hops were obtained. The operative conditions following Cicaloni et al. [12], with slight modifications. About 1 g of inflorescences and 1 g of powder were placed individually inside a 15 mL glass vial with PTFE-coated silicone septum. The collection of volatiles was carried out using an SPME device from Supelco (Bellefonte, PA, USA) equipped with 1 cm fiber coated with 50/30 µm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane). Before use, the fiber was conditioned at 270 °C for 30 min. The equilibration time for all hops samples was achieved heating to 50 °C for 10 min. At this point, the fiber was exposed to the headspace of the samples for 30 min at 50 °C to adsorb and concentrate the volatiles. Finally, the thermal desorption phase of the captured compounds was carried out by inserting the fiber into the GC injector maintained at 250 °C for 3 min in split mode.

2.5. GC-MS Analysis of Fresh Hops Samples

A Clarus 500 model Perkin Elmer (Waltham, MA, USA) gas chromatograph coupled with a mass spectrometer equipped with a FID (flame detector ionization) was used to carry out the analyses of all samples. In the oven GC, an Agilent VF-1 ms, (60 m × 0.32 mm ID, DF = 1.0 µm), nonpolar 100% dimethylpolysiloxane phase, capillary column was housed. To characterize the volatile composition of the hops samples, the oven conditions were set following Iannone et al. [13]. Briefly, the GC oven temperature started from 60 °C to 220 °C at 6 °C/min and was finally held for 15 min. Helium was used as the carrier gas at

a constant flow of 1 mL/min. MS scans were recorded within the range 40–500 m/z using EI ionization (energy 70 eV). Identification of compounds was based on the comparison of the mass spectra of pure components stored in the Nist 02 library database [14] and on the comparison of the Linear Retention Indices (LRIs) calculated using a series of alkane standards (C_8–C_{25} n-alkanes) with the available retention data reported in the literature (Chemistry Nist WebBook). The relative proportions of the constituents were expressed as percentages and were obtained by FID peak-area normalization (mean of three replicates) without the use of an internal standard and any factor correction. The analyses were carried out in triplicate.

2.6. DI-SPME-GC-MS and GC-MS Analysis ofHops Extracts

To determine the volatile content of methanolic hops extracts, 1 µL of both samples was injected manually at 270 °C into the GC injector with an injector split ratio of 1:20. To perform these analyses, the applied programmed temperature was initially at 50 °C, then was increased to 150 °C at 6 °C/min and held for 2 min, then increased to 250 °C at 8 °C/min and held for 20 min. Regarding the aqueous extracts, direct immersion solid phase microextraction (DI-SPME) was performed. In this case, after reaching the equilibrium phase, the fiber was immersed directly in the aqueous solution maintained at 50 °C for 20 min. The gas chromatographic analysis was carried out under the same operating conditions used for the analysis of the fresh material.

The mass spectrometer was operated under the same conditions used for the analyses of fresh hops samples, and the identification and quantification of the detected compounds were performed as reported above (Section 2.5). The analyses were carried out in triplicate.

2.7. GC-MS Analysis of Derivatized Methanolic Extracts

To describe the non-volatile content, 1 mg of each methanolic extract was added of 300 µL of pyridine and 100 µL of bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with heating at 80 °C for 30 min; 1 µL of the silylated sample was manually injected at 270 °C into the GC injector in the splitless mode. The analysis was performed using the same apparatus GC-FID/GC-MS. The oven temperature program was as follows: 50 °C, then a gradient of 6 °C/min to 150 °C for 1.0 min and a gradient of 8 °C/min to 250 °C for 20 min. Mass spectra were acquired in an electron ionization mode. The identification of compounds was based on the percentage of similarity plus comparison of mass spectra (MS) with the percentage of total ion chromatograms (TIC%)using software NIST data library. Relative percentages for quantification of the components were calculated by electronic integration of the GC-FID peak areas, and no response factors were calculated. The analyses were carried out in duplicate.

2.8. Determination of Total Polyphenols

The total polyphenolic content of the extracts was determined spectrophotometrically by the Folin–Ciocalteau method, using gallic acid as the reference compound and expressing the results as mg of gallic acid equivalents (GAE) per g of extract and mg GAE per g of fresh hops (leaves or inflorescences) [15]. Briefly, an appropriate aliquot of each extract was added to 50 µL of Folin–Ciocalteau reagent and, after 3 min, also 100 µL of a saturated sodium carbonate solution. The final volume of the reaction mixture (2.5 mL) was reached with distilled water and incubated in the dark for 1 h at room temperature. The absorbance was read at 725 nm using a UV-visible spectrophotometer (Jenway 6310, Keison, Chelmsford, Essex, UK). The test was performed in triplicate.

2.9. Determination of Total Flavonoids

The total flavonoid content of the extracts was determined by the aluminum chloride colorimetric method using quercetin as the reference compound and expressing the results as mg of quercetin equivalents (QE) per g of extract and mg QE per g of fresh hops (leaves or inflorescences) [11]. Briefly, 100 µL of each diluted extract was mixed with 300 µL of

methanol, 20 µL of 10% aluminum chloride and 20 µL of 1 M potassium acetate. The final volume of the reaction mixture (1 mL) was reached with distilled water and incubated in the dark for 30 min at room temperature. The absorbance was read at 420 nm using a UV-visible spectrophotometer (Jenway 6310, Keison, Chelmsford, Essex, UK). The test was performed in triplicate.

2.10. Antioxidant Assays

2.10.1. DPPH (2,2-Diphenyl-picryl hydrazyl) Test

The scavenging against the free radical was performed following Vitalini et al. [16]. Briefly, 2.45 mL of the DPPH· solution (0.35 g/L methanol), diluted with methanol to an absorbance of 1.00 (±0.03) units at 515 nm, was placed in a test tube; then, 50 µL of each extract was added. After 30 min of incubation in the dark at room temperature, the absorbance was read at 515 nm using a UV-visible spectrophotometer (Jenway 6310, Keison, Chelmsford, Essex, UK). The test was performed in triplicate. Results were expressed both as RSA (radical scavenging activity) % = $[(ABS_{control} - ABS_{sample})/ ABS_{control}] \times 100$ and as mM of Trolox Equivalents (TE). The assay was performed in triplicate.

2.10.2. ABTS [2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt)] test

The scavenging activity was determined following Vitalini et al. [17]. The $ABTS^{\cdot+}$ radical cation was obtained by reacting ABTS 7 mM with potassium persulfate 2.45 mM and keeping the mixture in the dark at room temperature for at least 6 h. Then, 1 mL of the $ABTS^{\cdot+}$ solution, diluted with ethanol to an absorbance of 0.7 (±0.02) units at 734 nm, was mixed for 30 s with 10 µL of each extract. The absorbance of the final reaction mixture was measured at 734 nm 20 s after the end of the mixing using a UV-visible spectrophotometer (Jenway 6310, Keison, Chelmsford, Essex, UK). The test was performed in triplicate. Results were expressed both as RSA (radical scavenging activity) % = $[(ABS_{control} - ABS_{sample})/ ABS_{control}] \times 100$ and as mM TE. The test was performed in triplicate.

2.11. Cell Cultures and Cytotoxicity Assay

Human non-small cell lung cancer (H1299) and melanoma (A375) cell lines were cultured in RPMI 1640 medium (Euroclone, Milan, IT, USA) supplemented with 10% fetal bovine serum (Hyclone, Thermoscientific, South Logan, UT, USA), 1% L-glutamine (Euroclone) and 100 µg/mL penicillin/streptomycin (Euroclone). A human breast cancer (MCF7) cell line was cultured in DMEM medium (LONZA, Verviers, Belgium) supplemented with 10% fetal bovine serum, 1% L-glutamine and 100 µg/mL penicillin/streptomycin. Cells were maintained at 37 °C in a humid 5% CO_2 environment. All the cell lines used were purchased from American Type Culture Collection (Manassas, VA, USA).

The two hops extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) at 50 mg/mL and then serially diluted in medium; 3×10^3 cells were cultured in a 96 well plate and, after 24 h, were treated with increasing concentrations (1–100 µg/mL) of the two extracts for 72 h. The effect of extracts on cell viability was evaluated by measuring 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) dye absorbance following manufacturer's protocol. Briefly, 20 µL of MTT reagent (5 mg/mL in PBS-(Euroclone)) was added to every well and, after 5 h, formazan crystals were solubilized in isopropanol (Sigma-Aldrich) and quantified by measuring absorbance at 540 nm. Cells were treated with <1% DMSO as vehicle control. Six technical replicates were run on the same plate, and the experiments were repeated at least in two biological replicates. IC_{50} values were calculated by using Graphpad Prism 6.

2.12. Statistical Analysis

All data were expressed as means ± standard deviation (SD). The data matrix was imported into MetaboAnalyst 5.0 online platform [18] and a fold change analysis was calculated by setting a fold change threshold equal to 2. Moreover, a VIP value was calculated. The VIP value represents the difference between the considered variables. A VIP

value above 1.0 indicated components that play an important role in differentiating between samples. Only components with VIP > 1.0 and $p < 0.05$ were selected as potential markers.

3. Results

3.1. Chemical Composition of Fresh Inflorescences and Leaves (Unpowdered and Powdered)

Using the SPME-GC-MS technique, thirty-six volatile compounds, listed in Table 1, were identified. In general, the chemical composition of the inflorescences was richer in components than that of the leaves. In fact, twenty-five and twenty-three molecules were detected in unpowdered and powdered inflorescences, respectively, compared to fifteen compounds found in both unpowdered and powdered leaves. Moreover, the quantity of sesquiterpenes was higher than that of monoterpenes in all the investigated samples. From a qualitative point of view, some compounds, such as 1-hepten-3-ol, styrene and α-terpineol, were found only in the leaves, while some fatty acid esters, such as, hexanoic acid, 5-methyl-, methyl ester, propanoic acid, 2-methyl-, 2-methylbutylester, propanoic acid, 2-methyl-, 3-methylbutylester, butanoic acid, 3-methylbutyl ester, 6-methylheptanoic acid, methyl ester, pentanoic acid, 3-methylbutyl ester, octanoic acid, methyl ester, geranic acid methyl ester and decanoic acid, methyl ester as well as some sesquiterpenes, such as, *α*-selinene, *γ*-cadinene, *δ*-cadinene and *γ*-gurjunene, were characteristic only of the inflorescences. The main quantitative difference between the two matrices concerned the *β*-myrcene content which ranged from 20.5% to 37.9% for the inflorescences, and from 1.9% to 2.7% for the leaves.

Table 1. Chemical volatile composition (percentage mean value ± standard deviation) of fresh hops inflorescences and leaves (unpowdered and powdered), as determined by SPME-GC-MS.

N°	COMPONENT [1]	LRI [2]	LRI [3]	Fresh I. [4] (Unpowdered)	Fresh L. [5] (Unpowdered)	Fresh I. [6] (Powdered)	Fresh L. [7] (Powdered)
1	1-hepten-3-ol	862	869	-	-	-	0.5 ± 0.02
2	styrene	893	898	-	1.3 ± 0.02	-	-
3	hexanoic acid, 5-methyl-, methyl ester	965	963	-	-	0.1 ± 0.02	-
4	*β*-pinene	988	986	-	-	0.9 ± 0.02	-
5	*β*-myrcene	991	987	37.9 ± 0.31	2.7 ± 0.02	20.5 ± 0.02	1.9 ± 0.02
6	propanoic acid, 2-methyl-, 2-methylbutylester	993	989	0.3 ± 0.05	-	-	-
7	propanoic acid, 2-methyl-, 3-methylbutylester	998	996	0.5 ± 0.02	-	0.9 ± 0.03	-
8	limonene	1028	1030	0.2 ± 0.02	-	0.4 ± 0.03	2.0 ± 0.03
9	trans-*β*-ocimene	1035	1040	0.1 ± 0.02	-	-	-
10	butanoic acid,3-methylbutyl ester	1052	1056	-	-	1.1 ± 0.02	-
11	6-methylheptanoic acid, methyl ester	1063	1068	0.2 ± 0.02	-	0.4 ± 0.03	-
12	pentanoic acid, 3-methylbutyl ester	1092	1090	0.1 ± 0.02	-	-	-
13	2-nonanone	1096	1092	0.2 ± 0.02	-	0.1 ± 0.02	-
14	octanoic acid, methyl ester	1128	1132	-	-	0.3 ± 0.02	-
15	α-terpineol	1175	1170	-	0.7 ± 0.01	-	0.1 ± 0.02
16	geranic acid methyl ester	1305	1302	0.2 ± 0.02	-	-	-
17	decanoic acid, methyl ester	1312	1309	-	-	0.2 ± 0.03	-
18	α-cubebene	1355	1350	0.2 ± 0.02	-	0.4 ± 0.03	0.3 ± 0.02
19	ylangene	1380	1376	0.2 ± 0.03	-	-	-
20	*α*-copaene	1384	1385	0.9 ± 0.04	3.1 ± 0.02	-	4.8 ± 0.03
21	*β*-caryophyllene	1442	1440	12.6 ± 0.03	20.2 ± 0.08	16.4 ± 0.04	71.6 ± 0.11

Table 1. *Cont.*

N°	COMPONENT [1]	LRI [2]	LRI [3]	Fresh I. [4] (Unpowdered)	Fresh L. [5] (Unpowdered)	Fresh I. [6] (Powdered)	Fresh L. [7] (Powdered)
22	humulene	1470	1465	28.4 ± 0.03	33.8 ± 0.02	35.3 ± 0.03	10.0 ± 0.10
23	γ-muurolene	1475	1471	2.6 ± 0.03	8.4 ± 0.02	2.7 ± 0.03	-
24	germacrene D	1480	1475	1.4 ± 0.02	2.5 ± 0.03	0.3 ± 0.03	0.9 ± 0.03
25	γ-gurjunene	1482	1477	0.2 ± 0.02	-	4.3 ± 0.03	-
26	β-eudesmene	1483	1480	2.9 ± 0.04	2.9 ± 0.02	1.6 ± 0.02	-
27	α-selinene	1492	1489	1.9 ± 0.03	-	-	-
28	γ-cadinene	1505	1509	1.6 ± 0.03	-	-	-
29	valencene	1520	1515	1.5 ± 0.04	6.5 ± 0.01	-	-
30	selina-3,7(11)-diene	1533	1530	1.4 ± 0.02	0.9 ± 0.02	1.6 ± 0.05	-
31	α-muurolene	1541	1537	-	3.8 ± 0.02	2.4 ± 0.01	2.8 ± 0.04
32	guaia-1(10), 11-diene	1555	1512	-	-	2.6 ± 0.01	0.8 ± 0.02
33	δ-cadinene	1559	1538	3.2 ± 0.05	10.3 ± 0.03	4.7 ± 0.03	3.4 ± 0.02
34	caryophyllene oxide	1591	1585		-	0.2 ± 0.03	-
	SUM			98.6	99.9	97.4	99.4
	Monoterpenoids			38.2	3.4	21.8	4.0
	Sesquiterpenoids			58.9	92.4	72.5	94.6
	Others			1.5	4.1	3.1	0.8

[1] The components are reported according to their elution order on an apolar column; [2] Linear Retention Indices measured on an apolar column; [3] Linear Retention Indices from the literature; [4] fresh inflorescences unpowdered components; [5] fresh leaves unpowdered components; [6] powdered inflorescences hops components; [7] powdered leaves hops components; not detected.

β-Caryophyllene and humulene were the most abundant compounds in all samples but followed a different trend. In detail, β-caryophyllene and humulene reached 20.2% and 33.8% in unpowdered leaves and 71.6% and 10.0% in powdered ones; in contrast, in the inflorescences, the percentage values were 12.6% and 28.4% in those unpowdered and 16.4% and 35.3% in those powdered. The chromatograms are shown in Figures S1–S4. Mass spectra of δ-cadinene and guaia-1(10), 11-diene are shown in Figures S13 and S14.

3.2. Data Analysis

To better understand which compounds are up- or downregulated between the two powdered/unpowdered datasets, we performed a fold change on fresh leaves and inflorescences. Figure 2A shows the important features selected by the fold–change analysis with a threshold of 2.0. The y-axis shows values based on the log2 scale, so that both upregulated and downregulated features can be plotted in a symmetrical way; the x-axis shows the compounds. The circle with Log2(FC) > 0, representing the feature above the threshold, was upregulated in powdered fresh leaves. Conversely, the circles with Log2(FC) < 0 represent the metabolites downregulated in powdered fresh leaves. Metabolites that were not significantly changed are shown by gray dots. The metabolites upregulated in powdered fresh leaves are only one (β-caryophyllene), whereas five compounds are downregulated. Similarly, Figure 2B shows the metabolite upregulated in powdered fresh inflorescences (pentanoic acid, 3-methylbutyl ester, 4-decenoic acid, methyl ester, Z- and γ-gurjunene) and two compounds downregulated (2-nonanone and germacrene D).

Additionally, a VIP plot, which indicates components that play an important role in differentiating between powdered/unpowdered fresh inflorescences and leaves was performed. The VIP value represents the difference between the considered variables. A VIP value above 1.0 indicated components that play an important role in differentiating between samples. In the case of fresh leaves (Figure 3A), the most important compounds were β-caryophyllene (more elevated in powdered in comparison to unpowdered) and humulene (more elevated in unpowdered in comparison to powdered). In the case of fresh leaves (Figure 3B), the most important compounds were β-myrcene (more elevated in unpowdered in comparison to powdered) and humulene (more elevated in powdered in comparison to unpowdered).

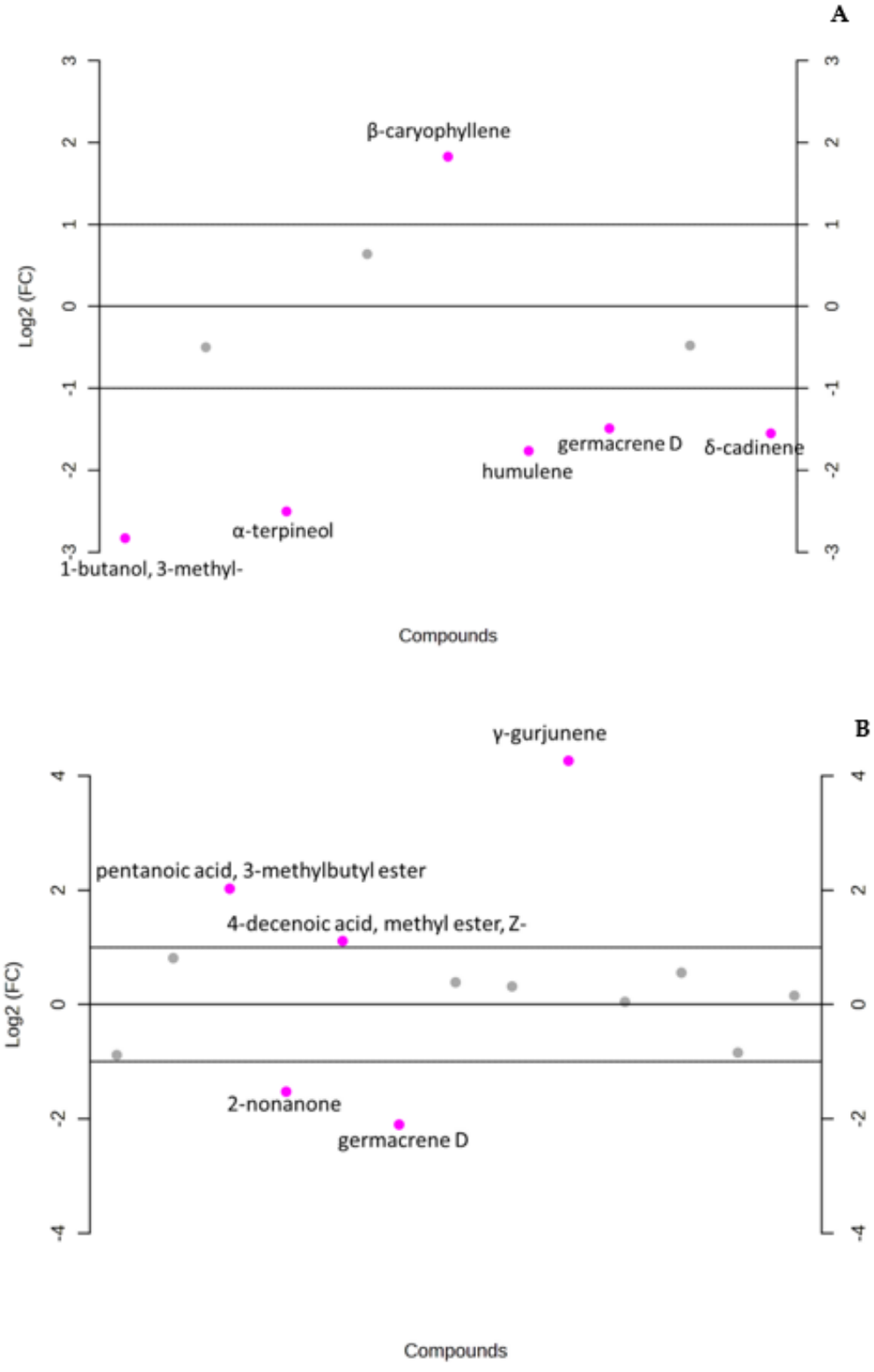

Figure 2. (**A**) Fold change of powdered/unpowered fresh leaves. The circle with Log2(FC) > 0, representing the feature above the threshold, is upregulated in powdered fresh leaves; the circles with Log2(FC) < 0 represent the metabolites downregulated in powdered fresh leaves; (**B**) fold change of powdered/unpowdered fresh inflorescences.

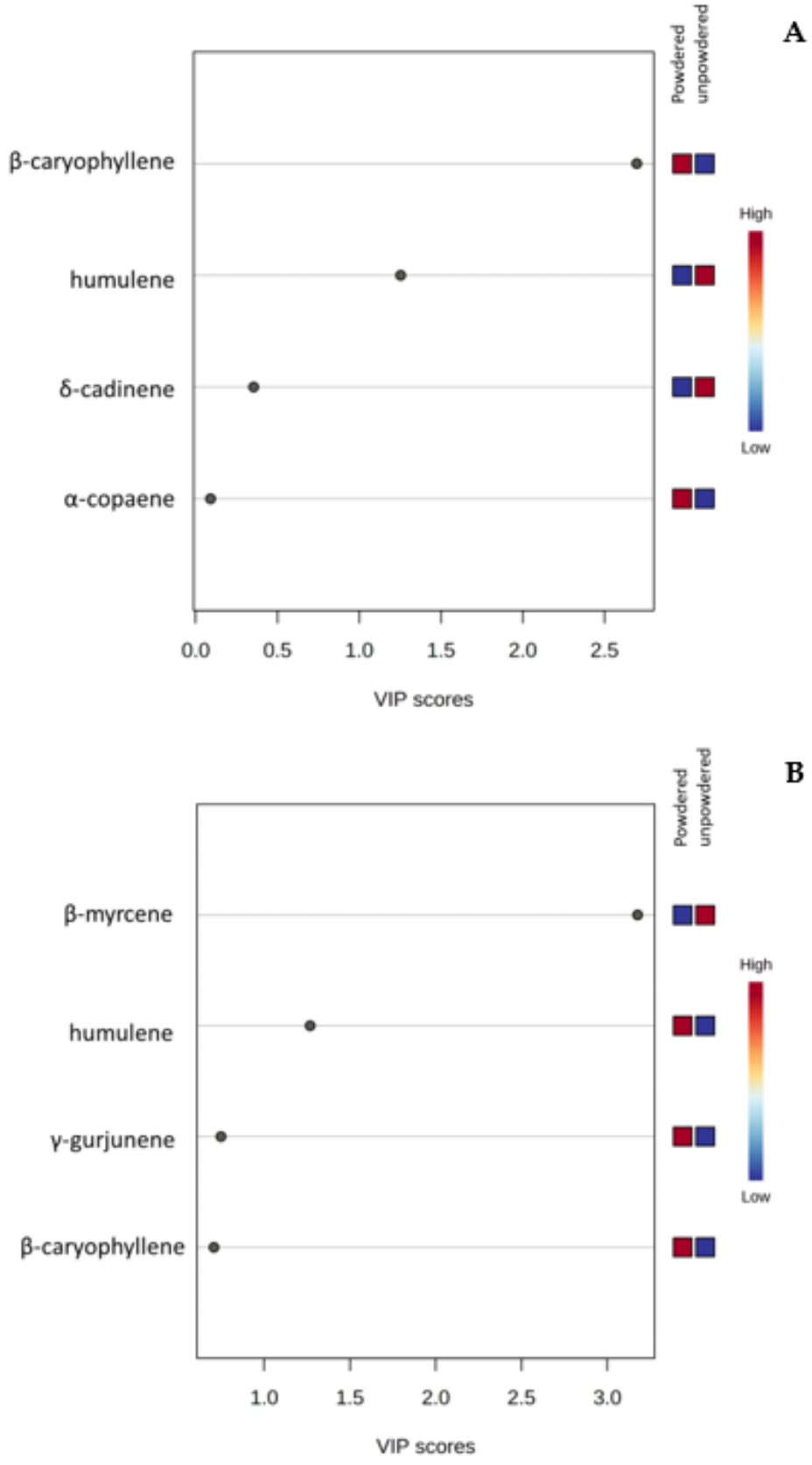

Figure 3. (**A**) VIP plot on powdered/unpowdered fresh leaves; (**B**) VIP plot on powdered/unpowdered fresh inflorescences.

3.3. Chemical Volatile Composition of Aqueous Extracts

The aqueous extracts were analyzed by the DI-SPME-GC-MS technique. In total, nine volatile compounds were found, of which seven were in fresh inflorescences (IAE) and five were in fresh leaves (LAE), (Table 2). The compounds detected in both matrices were the monoterpenes *β*-myrcene (13.1%; 8.3%), linalool (6.8%; 49.6%) and the sesquiterpene humulene (41.6%; 8.0%). Other sesquiterpene compounds, such as *β*-caryophyllene (10.8%), *δ*-cadinene (4.4%) and humulene epoxide II (14.3%), were present in IAE and missing in LAE. The chromatograms are shown in Figures S5 and S6.

Table 2. Chemical volatile composition (percentage mean value ± standard deviation) of aqueous extracts from hops inflorescences and leaves, as determined by DI-SPME-GC-MS.

N°	COMPONENT [1]	LRI [2]	LRI [3]	IAE	LAE
1	pentane, 1-methoxy-	710	708	-	17.5 ± 0.04
2	1-butanol, 3-methyl-	715	718	-	16.6 ± 0.03
3	*β*-myrcene	985	987	13.1 ± 0.05	8.3 ± 0.02
4	propanoic acid, 2-methyl-, 3-methylhetyl ester	995	996	8.9 ± 0.03	-
5	linalool	1089	1092	6.8 ± 0.03	49.6 ± 0.09
6	*β*-caryophyllene	1442	1440	10.8 ± 0.05	-
7	humulene	1490	1499	41.6 ± 0.08	8.0 ± 0.03
8	*δ*-cadinene	1561	1538	4.4 ± 0.02	-
9	humulene epoxide II	1572	1570 *	14.3 ± 0.03	-
	SUM			99.9	100.0
	Monoterpenoids			19.9	57.9
	Sesquiterpenoids			71.1	8.0
	Others			8.9	34.1

[1] The components are reported according to their elution order on an apolar column; [2] Linear Retention Indices measured on an apolar column; [3] Linear Retention Indices from the literature; * Kovats Index; IAE: fresh inflorescences hops aqueous extract components; LAE.: fresh leaves hops aqueous extract components; not detected.

3.4. Chemical Volatile Composition of Methanolic Extracts

The methanolic extracts were analyzed by direct injection in a GC-MS apparatus. In general, the number of compounds detected in the methanolic extract obtained from fresh inflorescences (IME) was higher than that found in the extract obtained from the leaves (LME). Furthermore, no compound in common to the two extracts was detected (Table 3). The components 2-heptanol, 2-methyl- (42.7%) and 2,3-butanediol (24.6%) were the most abundant in IME, while fatty acid derivatives, such as 9,12,15-octadecatrienal (47.6%), palmitic acid (23.2%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (11.8%), were the most abundant components found in LME. Also interesting was the presence of two furan derivatives, such as *trans*-arbusculone (1.9%) and 2(5H)-furanone, 5,5-dimethyl- (8.7%) in IME sample. The chromatograms are shown in Figures S7 and S8. Mass spectra of 9,12,15-octadecatrienal and 2-heptanol-2-methyl are shown in Figures S1 and S12.

Table 3. Chemical volatile composition (percentage mean value ± standard deviation) of methanolic extracts from hops inflorescences and leaves, as determined by GC-MS.

N°	COMPONENT [1]	LRI [2]	LRI [3]	IME	LME
1	butanal, 3-methyl-	648	651	-	2.5 ± 0.06
2	propanoic acid, 2-methyl-	762	765	3.9 ± 0.03	-
3	2,3-butanediol	770	769	24.6 ± 0.04	-
4	butanoic acid, 3-methyl	851	857	7.1 ± 0.02	-

Table 3. *Cont.*

N°	COMPONENT [1]	LRI [2]	LRI [3]	IME	LME
5	acetic acid 2-hydroxyethyl ester	866	862	-	2.7 ± 0.06
6	butanoic acid, 2-methyl-	871	868	1.2 ± 0.02	-
7	2(5H)-furanone, 5,5-dimethyl-	954	952	8.7 ± 0.04	-
8	2-pentenoic acid, 2-methyl-	974	974 *	3.7 ± 0.03	-
10	2-heptanol, 2-methyl-	990	920 *	42.7 ± 0.04	-
11	*trans*-arbusculone	1080	1071	1.9 ± 0.04	-
12	*trans*-nerolidol	1571	1547	1.3 ± 0.02	-
13	humulene epoxide II	1572	1570 *	0.8 ± 0.02	-
14	neophytadiene	1842	1836	-	8.8 ± 0.06
15	palmitic acid	1980	1973	-	23.2 ± 0.04
16	3,7,11,15-tetramethyl-2-hexadecen-1-ol	2112	2116	-	11.8 ± 0.04
17	9,12,15-octadecatrienal	2130	2109 §	-	47.6 ± 0.05
	SUM			95.9	96.5
	Monoterpenoids			-	-
	Sesquiterpenoids			2.1	-
	Others			93.8	96.5

[1] The components are reported according to their elution order on an apolar column; [2] Linear Retention Indices measured on an apolar column; [3] Linear Retention Indices from the literature; * Kovats Index; § normal alkane RI; IME: fresh inflorescences hops methanol extract components; LME: fresh leaves hops methanol extract components; not detected.

3.5. Chemical Composition of Methanolic Extracts after Derivatization

Direct injection analyses of silylated extracts allowed the identification of thirty-five components, of which twenty-seven in IME and eighteen in LME belonged to different chemical class, including organic acids, sugars, sugar alcohols and others (Table 4). In detail, eight different acids were found in IME and three in LME. Among them, lactic acid (2.8%) was the most abundant in IME, while acrylic acid (2.1%) was the most abundant in LME. Lactic and acrylic were the only two acids common to the two extracts.

Sugars represented the richest group of detected compounds. Fructofuranose (31.4% in IME; 31.9% in LME) was the one with the highest percentage values in both samples followed by glucopyranose (21.6%) and mannopyranose (15.3%) in IME and by tagatofuranose (18.6%) in LME.

Among the sugar alcohols, glycerol (4.2%; 6.8%) and pinitol (0.9%; 13.9%) were the most abundant in IME and LME, respectively. Lastly, a significant amount of linolenic acid (4.6%) in IME sample was also measured.

The chromatograms are shown in Figures S9 and S10.

3.6. Total Polyphenols and Flavonoids

The total polyphenol and flavonoid content of the Chinook hops inflorescences and leaves are shown in Tables 5 and 6, respectively. The polyphenols ranged from 203.6 to 374.7 mg GAE/g of extract, more abundant in both methanolic ones. LME contained less polyphenols than IME, while LAE was richer than IAE (Table 5).

Table 4. Chemical composition (percentage values) of methanolic extracts after derivatization from hops inflorescences and leaves, as determined by GC-MS.

N°	COMPONENT	LRI ^{calc}	LRI ^{lit}	IME	LME
		ORGANIC ACIDS			
1	lactic	1050	1060	2.8	0.4
2	4-hydroxymandelic	1775	1782	-	0.3
3	2-hydroxy-3-methylvaleric	1670	1680 §	tr	-
4	glycolic	1986	1997 §	0.1	-
5	glyceric	1355	1350	0.1	-
6	erytronic	1572	1567	0.3	-
7	galacturonic	2100	2096	0.2	-
8	quininic	1862	1854	1.2	-
9	acrylic	1110	1100	2.3	2.1
10	arabinonic	1814	1812	-	0.6
		SUGARS			
11	lyxose	1600	1610 *	tr	0.4
12	ribose	1660	1669 *	tr	-
13	xylose	1710	1740 §	tr	0.2
14	rhamnose	1630	1642 *	tr	-
15	ribofuranose	1625	1637 *	-	1.2
16	fructofuranose	1845	1857 *	31.4	31.9
17	sorbofuranose	1782	1779	9.3	3.2
18	tagatofuranose	1807	1800	-	18.6
19	mannopyranose	1785	1793	15.3	-
20	talopyranose	1935	1943 *	1.1	-
21	glucopyranose	1841	1837	21.6	-
22	allofuranose	1888	1896 *	1.2	-
23	talofuranose	1875	1882	0.3	-
24	galactopyranose	1935	1952 §	-	0.4
25	methyl β-arabinofuranoside	1820	1834 §	-	0.3
26	2-O-glycerol-α-galactopyranoside	2168	2180 §	0.4	-
		SUGAR ALCOHOLS			
27	glycerol	1305	1300	4.2	6.8
28	ribitol	1718	1727	0.1	0.3
29	arabitol	1770	1776	-	1.7
30	pinitol	1855	1869 *	0.9	13.9
31	myo-inositol	2090	2096	0.9	1.0
		FATTY ACIDS			
32	linolenic	2212	2218	4.6	-
		AMINOACIDS			
33	valine	1218	1221	0.1	-
		OTHERS			
34	phytol	2155	2162	0.8	-
35	acetoacetic acid, ethyl ester	938	944	-	11.9

IME: Percentage values of derivatized inflorescences methanolic extract components; LRI: Linear Retention Indices from the literature; * Kovats Index; § normal alkane RI; LME: percentage values of derivatized leaves methanolic extract components; tr: traces (mean value < 0.1%).

Table 5. Polyphenols of Chinook hops inflorescences and leaves.

Extracts	Total Polyphenols	
	(mg GAE/g Extract)	(mg GAE/g Fresh Hop)
IAE	203.6 ± 3.6	16.3 ± 0.4
LAE	295.3 ± 7.9	65.0 ± 2.3
IME	374.7 ± 6.9	40.7 ± 0.7
LME	317.5 ± 1.6	87.3 ± 1.5

IAE: Inflorescences aqueous extract; LAE: leaves aqueous extract; IME: inflorescences methanol extract; LAE: leaves methanol extract; GAE: gallic acid equivalents.

Table 6. Flavonoids of Chinook hops inflorescences and leaves.

Extracts	Total Flavonoids	
	(mg QE/g Extract)	(mg QE/g fresH Hop)
IAE	103.7 ± 6.2	8.3 ± 0.5
LAE	92.7 ± 5.7	20.0 ± 1.2
IME	85.6 ± 4.0	9.3 ± 0.3
LME	117.3 ± 3.2	32.25 ± 0.6

IAE: Inflorescences aqueous extract; LAE: leaves aqueous extract; IME: inflorescences methanol extract; LAE: leaves methanol extract; QE: quercetin equivalents.

The flavonoids varied between 85.6 and 117.3 mg QE/g of extract (Table 6). In this case, the differences between the four samples were much smaller. The extract in which the flavonoids are most abundant is LME, followed by IAE, LAE and IME. It is worth noting that the extract showing the highest level of polyphenols was found to be the poorest in flavonoids. The results obtained by calculating the polyphenol and flavonoid content for the fresh parts of hops showed a lower amount of these secondary metabolites in the inflorescences than in the leaves (Tables 5 and 6).

3.7. Antioxidant Activity

The results of the antioxidant activity of hops extracts displayed in Tables 7 and 8 were in line with their phenolic content. The greatest effects were observed for the methanolic extracts of inflorescences and leaves. They were able to inhibit the two radicals in a similar way with values close to 100% in the case of $ABTS^{\bullet+}$ and above 80% against $DPPH^{\bullet}$. Otherwise, the aqueous extracts proved to be less effective, albeit still with considerable activity. Only IAE showed an ability to inactivate DPPH below 20% (Table 8). Furthermore, the activity of IAE was halved compared to that of LAE toward the two radicals. As reported in Tables 7 and 8, the antioxidant activities by both assays were also expressed as mM Trolox equivalents.

Table 7. RSA% and mM Trolox values of Chinook hops extracts in ABTS assays.

Extracts	ABTS	
	RSA (%)	mM TE
IAE	36.2 ± 1.5	0.78 ± 0.1
LAE	62.9 ± 1.2	1.38 ± 0.0
IME	96.3 ± 0.8	2.14 ± 0.2
LME	96.1 ± 1.1	2.13 ± 0.8

IAE: inflorescences aqueous extract; LAE: leaves aqueous extract; IME: inflorescences methanol extract; LAE: leaves methanol extract; RSA: radical scavenging activity; TE: Trolox equivalents.

Table 8. RSA% and mM Trolox values of Chinook hops extracts in DPPH assays.

Extracts	DPPH	
	RSA (%)	**mM TE**
IAE	15.6 ± 1.1	0.05 ± 0.0
LAE	37.4 ± 0.7	0.13 ± 0.0
IME	84.8 ± 0.5	0.30 ± 0.0
LME	81.7 ± 1.0	0.29 ± 0.0

IAE: inflorescences aqueous extract; LAE: leaves aqueous extract; IME: inflorescences methanol extract; LAE: leaves methanol extract; RSA: radical scavenging activity; TE: Trolox equivalents.

3.8. Cytotoxic Activity

To explore the antitumor ability of the two methanolic extracts, human non-small cell lung cancer (H1299), melanoma (A375) and breast cancer (MCF7) cell lines were exposed to increasing concentrations (1–100 μg/mL) of leaf or inflorescence extracts for 72 h.

As shown in Table 9, the two extracts induced a comparable cytotoxic effect in the three different tumor histotypes. In particular, LME showed IC_{50} values ranging from 190 to 240 μg/mL, whereas the IME showed IC_{50} values ranging from 180 to 210 μg/mL.

Table 9. IC_{50} values of LME and IME in human cancer cell lines.

Cell Lines	LME (IC_{50}, μg/mL)	IME (IC_{50}, μg/mL)
H1299	220 ± 10	180 ± 20
A375	240 ± 25	210 ± 16
MCF7	190 ± 30	190 ± 22

IC_{50} values were calculated after 72 h exposure to the extracts.

4. Discussion

In this work, the powdered and unpowdered fresh inflorescences and leaves of Chinook hops were subjected to a thorough investigation for the characterization of their chemical profile using different techniques which allowed us to identify a large number of metabolites belonging to different chemical classes.

In terms of volatile content, the greatest number of works in the literature concern the characterization of dried hops inflorescences, while little has been conducted on the fresh material and even less on the leaves. Su et al. [19] identified β-myrcene, methyl octanoate, geraniol and linalool as major compounds in five fresh Cascade and Chinook hops harvested from different locations in Virginia, by gas chromatography–mass spectrometry–olfactometry (GC–MS–O) and aroma extract dilution analysis (AEDA). HS-SPME–GC–MS–O was also employed to compare the aroma profiles in five hops dried by different strategies. The obtained results revealed aroma content differences from three drying methods [20]. However, most of the work carried out on fresh hops was aimed at determining the content of α-acids. For example, Schindler et al. [21] quantified, by high performance liquid chromatography (HPLC), the resinous compounds in fresh Cascade cv. hops, while Tang and colleagues [22] identified the phenolic compounds in the dried hops pellets from Australia by an LC-ESI-QToF/MS technique.

Other papers showed the chemical volatile composition of essential oils (EOs) obtained from hops. EOs obtained from Cascade, Chinook and Comet grown in southern Italy were investigated to assess the adaptability of these American hops varieties to the Mediterranean environment. Myrcene, β-caryophyllene and α-humulene were the main components in all Eos, although Chinook EO differed from others for its sesquiterpene content [23]. In general, hops EOs chemical composition is responsible of the characteristic aroma of beer and the many positive effects on human health [24].

Regarding the studies conducted on the leaves, a metabolomic analysis by UPLC-QTOF-MS^E was carried out to determine the metabolite changes in the leaves of the plants of three hops cultivars (Cascade, Sultana, and the wild cv. Neomexicanus) subjected to varying degrees of drought stress. The results showed some chemicals, such as monoacylated lipid compounds, as markers of drought tolerance [25]. A similar study concerned the effect of water supply, in particular drought stress, on the content of xanthohumol, polyphenols and α-acids in the leaves and cones of nine hops cultivars grown in Slovenia in a pot experiment under three regimes with different water sources. The obtained data showed that the content of secondary metabolites depended more on the type of cultivar than different water source regimes [26].

A comparison of the chemical composition and antioxidant activity between leaves and inflorescences of hops was previously carried out. For example, Derkanosova et al. [27] reported the data on the aqueous extracts of Obyknovenny hops produced in Russia. In the two samples, the content of α-acids and total phenols was similar in terms of quality but different from a quantitative point of view with poorer leaf extract and less antioxidant power. Abram and co-authors [28] investigated cv. Aurora and cv. Hallertauer Magnum from four different European hop-growing regions in Slovenia, Austria, Germany and the Czech Republic, respectively. The inflorescences had both higher levels of phenols and greater radical scavenging ability in the DPPH assay. Despite this, the leaf extract from one of the two cultivars was found to be the best iron reducer. More recently, Keskin et al. [29] found that 99% methanol extract from hops inflorescences and leaves (unspecified cultivar) had similar total polyphenolic content and a good activity against DPPH. Dziedziński and collaborators [30] explored the feasibility of using Magnum hops leaves and inflorescences in varying percentages as functional components of teas. After preparation, the estimation of the total polyphenol content and free radical scavenging power demonstrated their richness in phenolic compounds and, in general, a greater capacity of tea containing hops leaves than those prepared with hops inflorescences in reducing the DPPH radical. The analysis of Lithuanian hops showed similar results in terms of total polyphenols and flavonoids and anti-radical activity between the four studied cultivars and some differences between their leaves and cones [31]. Liu et al. [32] confirmed the antioxidant activity of Chinook hops by testing the hot water, ethanol and CO_2 extracts obtained from the dried inflorescences by two complementary in vitro assays, such as inhibition of hydroxyl radicals and protection of a β-carotene-linoleic acid model system.

Any differences between the data on chemical profiles and related biological activity of these works and our obtained results on Chinook hops extracts can be reasonably attributed to the fresh and not dried or lyophilized starting plant material compared to the used solvent and/or to the extraction method, and the probable effects from both biotic (e.g., competitors, predators, parasites) and abiotic factors (physical or chemical such as soil, sunlight, temperature, water, salinity, oxygen, heavy metals) [33]. For example, in our previous work [11], the dried inflorescences of the same hops cultivar, also grown in Italy but in a different area, were analyzed, highlighting a composition rich in β-myrcene, β-caryophyllene and humulene. While for these last two compounds the percentage values were almost overlapping, the content of β-myrcene was significantly lower than that found in fresh inflorescences investigated in this study. A careful statistical investigation highlighted that for fresh leaves (Figure 3A), the most important compounds were β-caryophyllene (higher in powder than in unpowdered) and humulene (higher in unpowdered than in powdered). On the other hand, for fresh leaves (Figure 3B), the most important compounds were β-myrcene (higher in unpowdered than in powdered) and humulene (higher in powdered than in unpowdered).

In general, plant polyphenols, divided into two major groups, flavonoids and non-flavonoids, have antioxidant activity among their most important biological properties [34]. Likewise, terpenes, a large and varied class of molecules produced by numerous plants, can function as antioxidant compounds. This prerogative is exploited in the pharmaceutical and cosmetic fields [35], and the search for antioxidant compounds among natural terpene

compounds has significantly increased in recent years [36]. Among the volatile terpenes identified in the Chinook hops extracts, the fatty acids found in IME and LME were known to exhibit significant free radical scavenging activity [37]. Further, linalool and humulene that represented the most abundant compounds in LAE and in IAE, respectively, were defined as a natural source of antioxidants due to their proven high activity [38,39].

In this study, we also demonstrated that the two extracts induced a marginal effect on reducing breast tumor, melanoma and non-small cell lung cancer cell proliferation. This observation agreed with that indicating that hops seed extract concentrations ranging from 184 to 278 μg/mL were able to inhibit 50% of breast cancer (MCF-7), non-small cell lung cancer (NCI-H460) and cervical (HeLa) and hepatocellular (HepG2) carcinoma cell growth [40]. By contrast, Farag MA and colleagues [41] previously reported that hops resin extracts significantly reduced the proliferation of human prostate (PC3) and colon (HT29) cancer cell lines. However, these differences may be due to the genetic background of the tested cell lines and/or to the different cultivars and the type of used plant material for the experiments. We cannot exclude that for normal cells the toxicity could be higher than for cancer cells; thus, further experiments are needed to evaluate the effect of extracts from hops flowers and leaves on normal cells.

Regarding the anticancer activity, it has also been reported that linalool showed cytotoxic effects against T-47D epithelial cells, inducing them to undergo apoptosis and thus triggering cell death [42]. Equally significant was the cytotoxic effect caused by humulene against hepatocellular carcinoma cells (HCC), through the induction of mitochondrial apoptosis [43]. Some compounds found in our extracts have also been reported to have cytotoxic potential. Indeed, the 2(5H)-furanone derivatives were active towards non-small cell lung cancer cell line A549 [44], and some fatty acids had the potential to reduce proliferation and induce apoptosis in breast cancer cells [45,46]. Furthermore, sugar and sugar derivatives have numerous reported biological activities, including the anticancer one [47]. In this context, further analyses will be needed to better understand the antitumor efficacy of our matrices, fresh leaves and inflorescences of Chinook hop, never investigated until now, by expanding the panel of cancer cell lines derived from different human histotypes.

5. Conclusions

In this study, the chemical composition of the powdered and unpowdered fresh leaves and inflorescences of Chinook hops was investigated by different methodologies highlighting the presence of several secondary metabolites, among them, terpene compounds, fatty acids, sugars and others. Moreover, for the investigated matrices, a significant antioxidant power and a marginal cytotoxic effect were demonstrated. In conclusion, the obtained results allow us to consider both matrices as natural and alternative sources of bioactive molecules useful in the formulation of new functional and healthy products. The project, which also provides for the valorization of hops leaves, considered until recently only an unexploited agricultural by-product, fits well into a circular economy framework aimed at diversifying activities through the use of a resilient and sustainable plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10020091/s1, Figure S1: Chromatogram of unpowdered inflorescences determined by SPME-GC-MS; Figure S2: Chromatogram of powdered inflorescences determined by SPME-GC-MS obtained with a slight modification in the applied programmed temperature compared to the one described in the Section 2.5; Figure S3: Chromatogram of unpowdered leaves determined by SPME-GC-MS; Figure S4: Chromatogram of powdered leaves determined by SPME-GC-MS obtained with a slight modification in the applied programmed temperature compared to the one described in the Section 2.5; Figure S5: Chromatogram of aqueous extract from hop inflorescences determined by DI-SPME-GC-MS; Figure S6: Chromatogram of aqueous extract from hop leaves determined by DI-SPME-GC-MS; Figure S7: Chromatogram of methanolic extract from hop inflorescences determined by GC-MS; Figure S8: Chromatogram of methanolic extract from hop leaves determined by GC-MS; Figure S9: Chromatogram of methanolic extract after derivatization from hop inflorescences determined by GC-MS; Figure S10: Chromatogram of

methanolic extract after derivatization from hop leaves determined by GC-MS; Figure S11: Mass spectrum of 9,12,15-octadecatrienal; Figure S12: Mass spectrum of 2-heptanol-2-methyl; Figure S13: Mass spectrum of δ-cadinol; Figure S14: Mass spectrum of guaia-1(10), 11-diene; Figure S15: Puntato Pharm; Figure S16: Hop rhizome transplant; Figure S17: Planting layout and irrigation method; Figure S18: Late hop flowering phase; Figure S19: View of plants in full bloom; Figure S20: Hop inflorescence collection; Figure S21: Harvested hop with the presence of lupulin; Figure S22: Fresh material packaging; Table S1: Tentative determined compounds.

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