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Hop (*Humulus lupulus* L.) Specialized Metabolites: Extraction, Purification, Characterization in Different Plant Parts and In Vitro Evaluation of Anti-Oomycete Activities against *Phytophthora infestans*

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Abstract: Botanicals represent a promising source of metabolites in the search for new biofungicides. In this context, this study aimed at evaluating the in vitro anti-oomycete activity of hop (*Humulus lupulus* L.) extracts and metabolites against *Phytophthora infestans*, an oomycete causing late blight disease in Solanaceae. Crude hydro-ethanolic extracts and dichloromethane sub-extracts of different parts (cones, leaves, stems and rhizomes) were characterized by UHPLC-UV–MS and some cone specialized metabolites were purified by CPC and preparative HPLC. A commercial hop cone essential oil was also analyzed by GC–MS. All extracts succeeded in inhibiting mycelial growth and spore germination with morphological alteration of the mycelium. Extracts of leaves showed a significant anti-oomycete activity compared to the extracts of cones, stems, and rhizomes. Moreover, no difference was noticed between the crude hydro-ethanolic extract and the dichloromethane sub-extract activity, except for leaves, with the apolar sub-extract being more active than the crude one. The extracts of cones succeeded in inhibiting more *P. infestans* than the essential oil, which appeared to be the less active evaluated modality. Some purified prenylated phenolic compounds also inhibited *P. infestans* although copper sulfate, a mineral fungicide control, was still more active. This study highlights the potential use of hop by-products as biofungicides to manage *P. infestans*.

Keywords: *Humulus lupulus* L.; *Phytophthora infestans*; anti-oomycete activity; morphological alteration; specialized metabolites; phenolic compounds; by-products

1. Introduction

Hop (*Humulus lupulus* L.) is a climbing dioecious plant that belongs to the Cannabaceae family. It is mainly cultivated for its female inflorescences, named hop cones, which are used in brewing industry for their bittering, aromatic and preserving properties [1,2]. These properties are due to secondary metabolites synthesized by lupulin glands found in mature hop cones at the base of the bracts. Indeed, hop cones are mainly composed of terpenoids (essential oil compounds) and prenylated phenolic compounds including acylphloroglucinols, also named bitter acids (α- and β-acids), and prenylflavonoids [3]. Hop is also recognized as a medicinal plant thanks to its various biological properties, usually attributed to phenolic compounds [4–6]. Many studies refer to sedative [7], antispasmodic [8], estrogenic [9,10], antioxidant [4,11], antiproliferative [12,13] and anti-inflammatory properties [14,15]. In addition, hop constituents have been well-known for many years for their antimicrobial properties [5] including antibacterial [11,16–18], antiviral [19,20], antiparasitic [1,18] and antifungal activities [21,22]. In human health, Gram-positive bacteria were

shown to be more sensitive to hop extracts than fungi [1,23]. Some prenylated phenolic compounds, xanthohumol, 6-prenylnaringenin, and to a lesser extent α-acids, have little or no effect against human pathogenic fungi such as *Candida albicans*, *Fusarium oxysporum*, and *Mucor rouxianus.* However, they can inhibit the growth of the dermatophytic fungi *Trichophyton mentagrophytes* and *T. rubrum* [16,21,23,24]. In food industry, some authors showed that bitter acids are not active on *Saccharomyces cerevisiae* and *S. pastorianus*; they can therefore be used to safeguard cultured yeast strains that are vital for beer production, while avoiding bacterial contamination [25]. By contrast, hop extracts can inhibit the growth of some *Aspergillus* and *Penicillium* strains making it possible to use them as natural preservatives in bread making [21,26]. Other studies underlined the antifungal potential of hop extracts and some of their metabolites against plant fungal pathogens [22,27,28]. The antifungal activity of different vegetative parts of hop was previously tested against the wheat pathogen *Zymoseptoria tritici*. Desmethylxanthohumol and co-humulone were identified as the main bioactive compounds of a crude hydro-ethanolic extract of cones, whereas the essential oil from cones showed a synergistic action in association with a synthetic fungicide [6].

Although the composition of cones is well-known and studied in the literature for their activities, few studies deal with hop by-products such as leaves [11,29] and stems [30], often considered as wastes. Indeed, leaves and stems represent the major biomass of hop production (nearly 75%) [29] and they remain unexploited by-products [6,11,30,31] that can be recovered in a circular economy perspective. [6].

Phytophthora infestans (Mont.) de Bary is the causal agent of late blight in solanaceous plants. This plant disease is one of the most devastating potato diseases worldwide [32,33]. Under favorable conditions, late blight can destroy the whole plot [34,35], which makes it an economic threat for potato production [33,36]. Due to late blight, annual crop losses, including costs of control and damage, were estimated at more than 3 billion dollars worldwide [34] and around 900 million dollars in Europe [37]. This pathogen is a heterothallic (A1 and A2 mating types) and hemibiotrophic fungus-like microorganism that belongs to the oomycetes class [34]. Currently, among all available management techniques (resistant cultivars, cultural methods, chemical control), the use of fungicides still prevails over other methods. Fungicides are used to control the development of *P. infestans* on aerial plant parts, up to 20 treatments per year in some cases [38]. However, intensive use of these fungicides is causing not only the appearance of resistant strains but also environmental pollutions [37,39].

Nevertheless, chemical fungicides are not the only problematical products. Copper-based products represent another concern, especially in organic productions that create ecological, health, and economic issues [40]. Even if copper has been prohibited in some northern European countries [40], the European authorization has been renewed for 7 years with the use of 4 kg/ha/year (Commission Implementing Regulation (EU) 2018/1981) starting from the beginning of 2019. Nonetheless, in vitro studies have been carried out on botanicals with the aim of finding a potential substitute for copper products or fungicides which are systematically used to manage *P. infestans* infections. Many studies focus on the activity of plant extracts or essential oils on mycelial growth [41–43] or germination [44–46] of the pathogen.

This study aimed at characterizing essential oil from hop cones by GC–MS as well as extracts from four parts (cones, leaves, stems, and rhizomes) by UHPLC-UV–MS. Their in vitro anti-oomycete activity was evaluated against the pseudo-fungus *Phytophthora infestans*. Afterwards, the main prenylated phenolic compounds were purified by CPC and their activity was determined and compared to copper sulfate as a reference active substance.

2. Materials and Methods

2.1. Preparation of Extracts, Sub-Extracts, and Purified Compounds of Hop

2.1.1. Extraction

Whole female plants of the Nugget hop cultivar were harvested at the maturity stage in the French Flanders (Beck farm, Bailleul, France) in 2017. Each part of the plant was collected: cones, leaves, stems, and rhizomes. The plants were dried in the dark at room temperature for 10 days. Each part was grounded independently with a blender and kept in the dark.

A solid/liquid extraction was rapidly carried out on grounded plant material. Crude hydro-ethanolic extracts (CHE) were obtained by four successive macerations stirred in the dark with ethanol/water (9:1, *v*/*v*). Ethanol (VWR Prolabo®, Fontenay-sous-Bois, France) was evaporated with a rotary evaporator (Heidolph™, Schwabach, Germany) and the CHEs were then freeze-dried (Telstar Cryodos™, Barcelona, Spain). The yields on a dry weight basis of each crude extract were: 25.6% (cones), 8.8% (leaves), 8.4% (stems) and 16.8% (rhizomes) dw.

CHE were then subjected to a liquid/liquid extraction with water/dichloromethane (DCM) (5:5, *v*/*v*) to obtain two immiscible phases. Anhydrous sodium sulfate (Na_2SO_4) was added to the organic phase to remove water traces. After filtration, DCM (VWR Prolabo®, Fontenay-sous-Bois, France) was evaporated to obtain a DCM sub-extract (DSE) for each part. The percentage yields obtained for each DCM sub-extract (cones, leaves, stems, and rhizomes) were respectively 39%, 31%, 17.6%, 7.7% dw.

2.1.2. Purification of Xanthohumol and the Mixtures of α- and αβ-Acids by CPC for Bioassays

Xanthohumol was purified from the DCM sub-extract of cones in one step by centrifugal partition chromatography (CPC) on an Armen instrument 250 mL rotor (SCPC-250-L) provided by Gilson® (Saint-Avé, France) and connected to a Shimadzu® pump (LC-20AP, Kyoto, Japan) [18]. All organic solvents for CPC were High Pressure Liquid Chromatography (HPLC)-grade except for the n-heptane, which was synthesis grade (Carlo Erba Reagents®, Cornaredo, Italy). Ethyl acetate (EtOAc) and methanol (MeOH) were purchased from Fisher Scientific® (Hampton, NH, USA). Water was purified using a Millipore Integral 5 (Merck®, Trosly-Breuil, France) water purification system with a resistivity of not less than 18 MΩ·cm^{-1}. Arizona solvent system P: n-heptane/EtOAc/MeOH/water (6:5:6:5; *v*/*v*) was selected. The analysis was monitored using a DAD detector (SPD-M20A). The CPC was first filled with the aqueous stationary phase in the ascending mode, introduced at 30 mL·min^{-1} at 500 rpm, and then incrementally increased to 1600 rpm. The organic mobile phase was then pumped into the column at a flow rate of 8 mL·min^{-1} and the extract was immediately injected after the displacement of the stationary phase was observed (approximatively 80 mL). Prior to injection, DSE of cones (1 g) was dissolved in 10 mL of equal parts organic and aqueous phases and filtered through a Millipore (0.45 µm) syringe filter. Fractions of 8 mL were collected every minute. The CPC was run in ascending mode for 60 min and then switched to extrusion mode for an additional 10 min at 30 mL·min^{-1} with 1600 rpm. The follow-up of CPC was monitored by online UV absorbance measurements at 330 nm and 370 nm. All the fractions were then characterized by TLC developed with toluene/ethyl acetate/formic acid (73:18:9; *v*/*v*) and then pooled into 5 sub-fractions (MC1 to MC5) from the ascendant mode and 3 sub-fractions (MC6 to MC8) from the extrusion mode, according to their chemical profiles. This CPC method allowed us to purify xanthohumol with 98% purity from MC4, as well as a mixture of α- and β-acids from MC2. The mixture of α-acids was obtained from MC2 by preparative HPLC according to the method described in 2.1.3.

2.1.3. Purification and Structural Identification of Major Acylphloroglucinols Obtained by Preparative HPLC for Characterization in Hop Extracts and Quantification

Major acylphloroglucinols were purified from MC2 using preparative HPLC-UV according to a protocol adapted from Bocquet et al. [18]. Preparative HPLC was carried out using a Shimadzu® HPLC system equipped with a LC-20AP binary high-pressure pump, a CBM-20A controller, and an SPD-M20A diode array detector. The software used was LabSolution Version 5.3. The stationary phase was a VisionHT HL C18 (5 µm, 250 × 22 mm) column (Grace®, Epernon, France). The mobile phase was composed of water (solvent A) and acetonitrile (solvent B). Acetonitrile (LC-MS grade) was purchased from Carlo Erba Reagents® (Val-de-Reuil, France). The following proportions of solvent B were: 10–75% (0–5 min), 75% (5–30 min), 75–100% (30–35 min), and 100% (35–45 min) at 12 mL·min^{-1}. A total of 500 µL of 60 mg·mL^{-1} fraction solubilized in methanol was injected. This process allowed us to purify acylphloroglucinols (co-humulone, humulone, ad-humulone, co-lupulone, lupulone, ad-lupulone) from the sub-fraction with a purity greater than 95%. The structure of these compounds was determined by comparison of NMR data and mass spectra with reported values previously obtained in our laboratory [18]. Humulone and lupulone were obtained in sufficient quantities for quantification. The products were stored under nitrogen in the dark at −20 °C.

2.2. Phytochemical Analyses

2.2.1. Characterization of Hop Commercial Essential Oil by GC-MS

Essential oil from hop cones Herbo Arôma™ (HE0518163) was obtained from Bardou herbalist (Aix les Bains, France). It was analyzed by gas chromatography coupled with mass spectrometry (GC–MS). A Varian CP-3800 with a capillary column VF-1ms (30 × 0.25 mm) and a mass-spectrometry Varian Saturn 2000 (Varian S.A., Les Ulis, France) (ion trap in full scan mode from 40 to 650 m/z) were used. The program started with 40 °C for 5 min, then the temperature was increased by 2 °C per minute to reach 200 °C for 5 min. The program finished at 250 °C for 10 min. The sample was solubilized at a concentration of 1% in hexane and 1 µL was injected using helium as a vector gas. Compounds were identified using some standards (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and the NIST (National Institute of Standards and Technology) database.

2.2.2. UHPLC-UV-MS Analyses and Quantification

Quantification studies and Ultra-High Performance Liquid Chromatography (UHPLC)-UV-MS analyses were performed on an Acquity UPLC® H-Class Waters® system (Waters, Guyancourt, France) coupled with a Diode Array Detector (DAD) and a QDa ESI-Quadrupole Mass Spectrometer. The software was Empower 3. Separation was achieved using a Waters® Acquity BEH C18 column (pore size 300 Å, particle size 1.7 µm, 2.1 × 150 mm, Waters, Milford, MA, USA) connected to a 0.2 µm in-line filter. The column was kept at 30 °C with a flow rate at 0.3 mL·min^{-1}. Compounds were eluted using the following gradients: 50% B (0–1 min), 50–75% B (1–3 min), 75% B (3–5 min), 75–100% B (5–7 min) and 100% B (7–9.5 min) with mobile phase A being water with 0.1% formic acid (v/v) and mobile phase B being acetonitrile with 0.1% formic acid (v/v). Total ion chromatograms were obtained in negative mode with a range of m/z 100–1000. Cone voltage and capillary voltage were, respectively, 10 V and 0.8 kV. Probe temperature was 600 °C.

Solutions of CHE and DSE were prepared in triplicate the same day in MeOH at 100 µg·mL^{-1} for cones and 1 mg·mL^{-1} for the other hop parts. The main chalcones and acylphloroglucinols were identified in CHE and DSE based on their retention time and their mass spectra, by comparison with purified standards.

The validation of the quantification method of xanthohumol and acylphloroglucinol derivatives was based on The International Conference for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use guideline Q2-R1 [47].

Quantification was achieved in UV at 370 nm for xanthohumol and 330 nm for acylphloroglucinols. From the corresponding calibration curves of the n-acids (humu-

lone for α-acids and lupulone for β-acids), co- and ad-acids were also quantified based on molecular weight ratios. External calibration curves of xanthohumol, humulone, and lupulone injected simultaneously at several concentrations were prepared to cover the expected range of concentrations in samples after preliminary injection of CHE and DSE solutions. Nine concentration levels for xanthohumol and lupulone, and twelve for humulone, were selected from fifteen working solutions (100 μg·mL^{-1} to 2.5 ng·mL^{-1}). This range of concentrations was prepared from a stock solution at 1 mg·mL^{-1} in MeOH stored at −20 °C. Calibration curves were built by plotting the peak area (y) as a function of the nominal concentration for each calibration level (x) and then fitted by weighted (1/x) least square linear regression. Linearity and sensitivity of the method were determined and reported in Table 1. The linearity was evaluated by plotting the peak area versus the concentrations of the compounds. Limit of Detection (LOD) was defined as a response of at least 3 times the level of noise, whereas Limit of Quantification (LOQ) was determined as the lowest concentration with a deviation <20% on back calculation. Intra and inter-day precisions were also determined from 2 μL of cone CHE solutions injected 3 times. They were prepared on three different days, in triplicate each day (n = 3, k = 3).

Table 1. Linearity and sensitivity of the quantification method from xanthohumol, humulone, and lupulone using UPLC-UV.

Products	LOD (ng·mL^{-1})	LOQ (ng·mL^{-1})	Linearity Range (μg·mL^{-1})	Equation	R^2
Xanthohumol	2.5	10	0.010–10	*y = 41129.80 x + 159.37*	0.9974
Humulone	10	25	0.025–100	*y = 7319.23 x + 41.38*	0.9975
Lupulone	10	100	0.025–10	*y = 9747.26 x + 46.84*	0.9979

2.3. In Vitro Anti-Oomycete Activity

2.3.1. *Phytophthora infestans* Culture Condition

The *P. infestans* strain was bought from MUCL Belgium (strain 54355, Ottignies-Louvain-la-Neuve, Belgium). It was maintained on a V8 medium (200 mL of V8 vegetable juice, 2 g of $CaCO_3$, 0.05 g of β-sitosterol and 15 g of agar per liter) at 18 °C in the dark.

2.3.2. Activity on Mycelial Growth

The inhibitory activity on mycelial growth was evaluated on agar medium, using the supplemented media technique from Andrivon [48] adapted in twelve-well plates (Cellstar®, Greiner Bio-one GmbH, Frickenhausen, Germany). Each extract or compound (CHE, DSE, purified compounds, and $CuSO_4$) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and then added to V8 medium. Two milliliters were poured per well and three repetitions per concentration were made. Discs of 0.4 cm were cut from the growth area of a ten-day-old *P. infestans* culture, grown at 18 °C in the dark on a Petri dish (90 mm) filled with 15 mL of V8 medium. Mycelial discs were transferred in the middle of the well with the mycelium on top of the disc. Plates were then sealed with parafilm and placed at 18 °C in the dark for 3 days. Two diameters per well were finally measured thanks to a ruler, which allowed us to determine mycelial growth.

Essential oil was tested separately at six concentrations ranging from 1200 to 20,000 mg·L^{-1}. All extracts and compounds were tested at eight concentrations, with a maximum of 1% (*v*/*v*) DMSO. First, the activity of CHE and DSE from separated plant parts was determined. For all CHEs, as well as the DSE of cones, stems and rhizomes, concentrations were tested between 4 and 500 mg·L^{-1}. For the DSE of leaves, concentrations tested ranged from 2 to 250 mg·L^{-1}. Xanthohumol was tested at concentrations between 4 and 500 mg·L^{-1} whereas α- and αβ-acids concentrations were between 2 and 250 mg·L^{-1}. The activity of purified compounds was compared to copper sulfate ($CuSO_4$, Merck®, Darmstadt, Germany) which was tested at eleven concentrations from 0.5 to 500 mg·L^{-1}.

2.3.3. Activity on Spore Germination

The inhibition of spore germination was evaluated by a 96-well plates (Corning® 3595, Corning Incorporated, New York, NY, USA) assay, using a protocol adapted from Sharma et al. [49]. Briefly, each extract (DSE or CHE) or pure compound (including copper sulfate as a positive control) was dissolved in DMSO and diluted with glucose peptone liquid medium (14.3 g of glucose and 7.1 g of bactopeptone per liter) in a range of concentrations (between 15.6 and 1000 mg·L^{-1} for CHE, DSE, bitter acids and between 7.8 and 500 mg·L^{-1} for copper sulfate and xanthohumol), with a maximal DMSO concentration of 1% (v/v) in the final volume. Essential oil was tested similarly but from 70 to 27,000 mg·L^{-1}. In each well, 112 µL of these solutions was poured, and 38 µL of glucose peptone (control for the net optical density: OD) or spore suspension was added to reach a final volume of 150 µL. For each concentration tested, controls were done in duplicate and extracts or compounds were replicated in 4 other wells. The spore suspension was obtained from sporangia that were recovered on glucose peptone from Petri dishes placed 18 days at 20 °C followed by 2 days at 10 °C, and then filtered. The sporangia suspension was calibrated at 1×10^5 sporangia.mL^{-1} and placed one hour at 4 °C before the beginning of the microplate assay. After adding the calibrated spore suspension, the plates were sealed and incubated at 20 °C in the dark under agitation at 140 rpm for 6 days. The OD values were read at 630 nm with a spectrophotometer (Biotek EL 808, BioTek Instruments, Santa Clara, CA, USA).

2.3.4. Microscopical Observations

Microscopical observations were done 24 h after germination evaluation under an optical microscope Nikon Eclipse 80i equipped with a Nikon Digital Camera Ddxm1200c (Nikon France S.A., Champigny sur Marne, France). Five microliters of glucose peptone and mycelium were taken from microwells for each extract or product (CHE and DSE extracts, purified compounds and $CuSO_4$) with three replicates each, and placed on glass slides with 5 µL of lactophenol blue solution. Samples were compared to the untreated control.

2.3.5. IC_{50} and Statistical Data Analysis

Statistical analysis was performed using R-software (R version 3.5.3, R Core Team, 2019) and its package nlstools. The half maximal inhibitory concentrations (IC_{50}), the concentration that inhibits the mycelial growth and germination by 50%, were determined by mycelial diameter measurements and OD values, using nonlinear regression with four parameters. One IC_{50} per extract or product was obtained from two independent experiments as described by Muchembled et al. [50]. A Fisher test with p-value (α = 5%) was used to determine if there were any differences between extracts or products. Then, a pairwise comparison of IC_{50} was performed based on confidence intervals with Bonferroni adjustments.

3. Results

3.1. Phytochemical Analyses

3.1.1. Characterization of the Essential Oil from Hop Cones by GC-MS Analysis

The essential oil from hop cones was mainly composed of three compounds: myrcene, *trans*-caryophyllene and α-humulene (Supplementary Figure S1).

These major compounds represent around 80% of the composition of the essential oil. More than 60% of the content corresponds to the two sesquiterpenes: *trans*-caryophyllene (33%) and α-humulene (31.4%). Myrcene (or β-myrcene), a common monoterpene, is the third main compound (16.4%). Six other compounds with levels greater than 1% were also identified (Table 2). This composition is in agreement with the literature [23].

Table 2. Compounds with levels greater than 1% in the commercial essential oil from hop cones.

Compound	Molar Mass (g·mol^{-1})	Chemical Formula	Retention Time (min)	Percentage in the EO
Myrcene	136	$C_{10}H_{16}$	16.8	**16.4%**
Coapene	204	$C_{15}H_{24}$	42.8	1.0%
***trans*-caryophyllene**	204	$C_{15}H_{24}$	45.3	**33.0%**
α-humulene	204	$C_{15}H_{24}$	47.3	**31.4%**
γ-muurolene	204	$C_{15}H_{24}$	48.6	1.3%
α-selinene	204	$C_{15}H_{24}$	49.1	1.1%
δ-cadinene	204	$C_{15}H_{24}$	51.3	1.2%
Humulene epoxide	220	$C_{15}H_{24}O$	55.5	1.1%
Cembrene	272	$C_{20}H_{32}$	73.4	2.4%
Compounds <1%	-	-	-	11.3%

3.1.2. Characterization and Quantification of Hop Phenolic Compounds from Cones, Leaves, Stems, and Rhizomes

To gain insight into the anti-oomycete activity of different hop parts, a phytochemical investigation was conducted on crude hydro-ethanolic extracts (CHE) and DCM sub-extracts (DSE) of the Nugget cultivar used for bioassays. Major prenylated chalcones and acylphloroglucinols were first identified by UHPLC-UV–MS in CHE and DSE based on their retention times and their mass spectra (Figure 1). They were then quantified by UHPLC-UV in the extracts thanks to the standards purified in our laboratory by CPC and preparative HPLC from the DSE of cones [18].

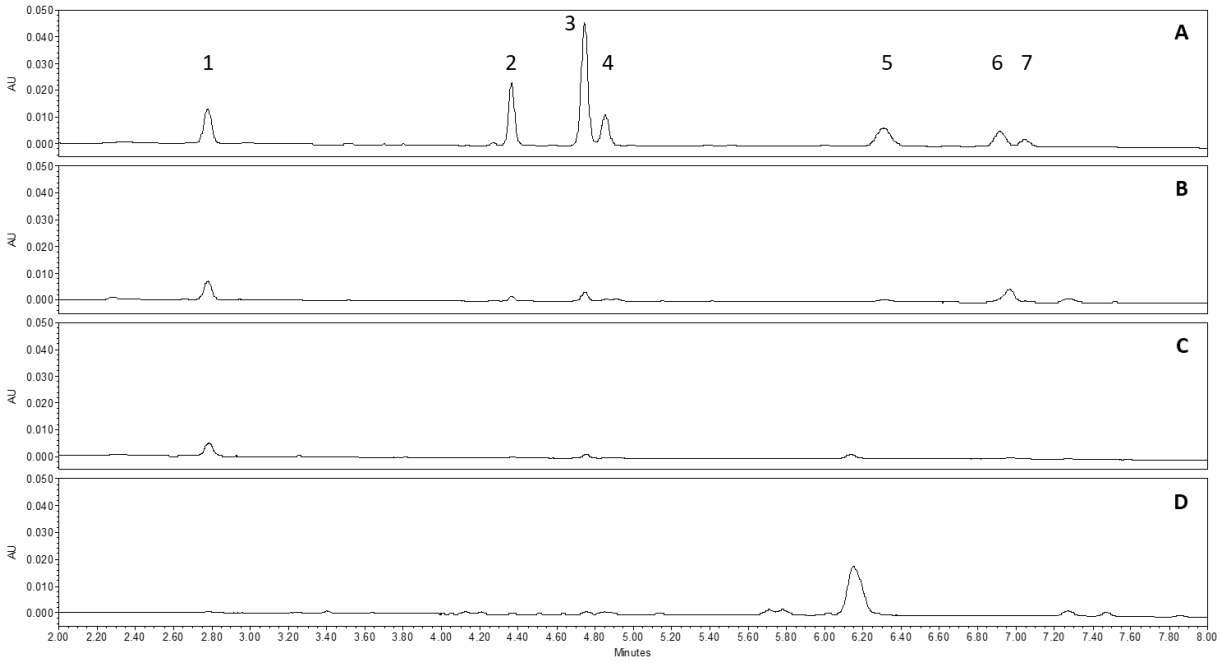

Figure 1. UHPLC-UV chromatograms (330 nm) of DCM sub-extracts (DSE) prepared at 100 µg·mL^{-1} in MeOH for (**A**) cones, and 1 mg·mL^{-1} in MeOH for (**B**) leaves, (**C**) stems and (**D**) rhizomes. Identified compounds and their retention time (rt) are: xanthohumol (1) rt 2.78 min, co-humulone (2) rt 4.37 min, humulone (3) rt 4.75 min, ad-humulone (4) rt 4.86 min, co-lupulone (5) rt 6.31 min, lupulone (6) rt 6.92 min, ad-lupulone (7) rt 7.05 min.

The method used showed a good linearity for each compound over the concentration range used for calibration. In addition, acceptable intra and inter-day precisions for xanthohumol (RSD % = 10.6, 12.0), humulone (RSD % = 12.3, 13.89) and lupulone (RSD % = 10.37, 11.86) were observed for the CHE of cones.

As previously highlighted [18], the CHE of cones showed the highest content of prenylated phenolic compounds in comparison with the CHE from other parts (Table 3, Supplementary Figure S2). No prenylated phenolic compounds could be quantified in the extract of rhizomes (values below LOD) and only xanthohumol and humulone were quantified in the extract of stems. Moreover, humulone was the main compound in the extract of cones (147 $\mu g \cdot mg^{-1}$), with a total α/β-acid (humulone/lupulone derivatives) ratio of 2.4, whereas lupulone was the most abundant compound in the extract of leaves, with a ratio of 0.5.

Table 3. Content (in $\mu g \cdot mg^{-1}$) of prenylated chalcones and acylphloroglucinols in (A) Crude hydro-ethanolic extracts (CHE) and (B) DCM sub-extracts (DSE) of different hop parts (n = 3, mean ± SD).

	Xanthohumol	Co-Humulone	N-Humulone	Ad-Humulone	Co-Lupulone	N-Lupulone	Ad-Lupulone
				Crude Hydro-Ethanolic Extracts (CHE)			
Cones	19.475 ± 1.738	64.452 ± 6.000	146.784 ± 13.715	41.584 ± 3.824	46.806 ± 4.173	32.053 ± 2.824	13.524 ± 1.195
Leaves	1.084 ± 0.126	0.674 ± 0.142	1.741 ± 0.211	0.556 ± 0.071	1.851 ± 0.240	4.290 ± 0.527	0.676 ± 0.091
Stems	0.106 ± 0.009	<LOQ	0.066 ± 0.017	<LOD	<LOQ	<LOQ	<LOQ
Rhizomes	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
				DCM sub-extracts (DSE)			
Cones	19.017 ± 0.546	71.232 ± 2.956	162.368 ± 6.608	45.805 ± 1.772	35.247 ± 1.212	24.189 ± 1.182	10.191 ± 0.441
Leaves	1.029 ± 0.051	0.542 ± 0.030	1.379 ± 0.066	0.401 ± 0.017	0.461 ± 0.021	2.335 ± 0.150	0.161 ± 0.011
Stems	0.685 ± 0.021	0.166 ± 0.007	0.590 ± 0.005	0.101 ± 0.003	<LOD	0.268 ± 0.011	<LOQ
Rhizomes	0.062 ± 0.004	0.077 ± 0.006	0.307 ± 0.018	0.374 ± 0.043	<LOD	<LOQ	<LOQ

LOD = Limit Of Detection; LOQ = Limit Of Quantification.

The liquid/liquid extraction with DCM tends to eliminate more polar compounds and to concentrate more α-acids. However, it has a lesser impact on xanthohumol levels except in stems and rhizomes. The partitioning even tends to slightly decrease the β-acid content, which can be explained by the sensitivity of these molecules to oxidation [51,52] (Table 3).

3.1.3. Characterization of xanthohumol, α-Acids, and αβ-Acids Mix by UHPLC-UV-MS

As the levels of phenolic compounds are higher in cones than in leaves, xanthohumol, the α-acids mix and the αβ-acids mix were more easily extracted from cones by CPC and preparative HPLC. Xanthohumol and the mix were characterized by UHPLC-UV–MS (Figure 2).

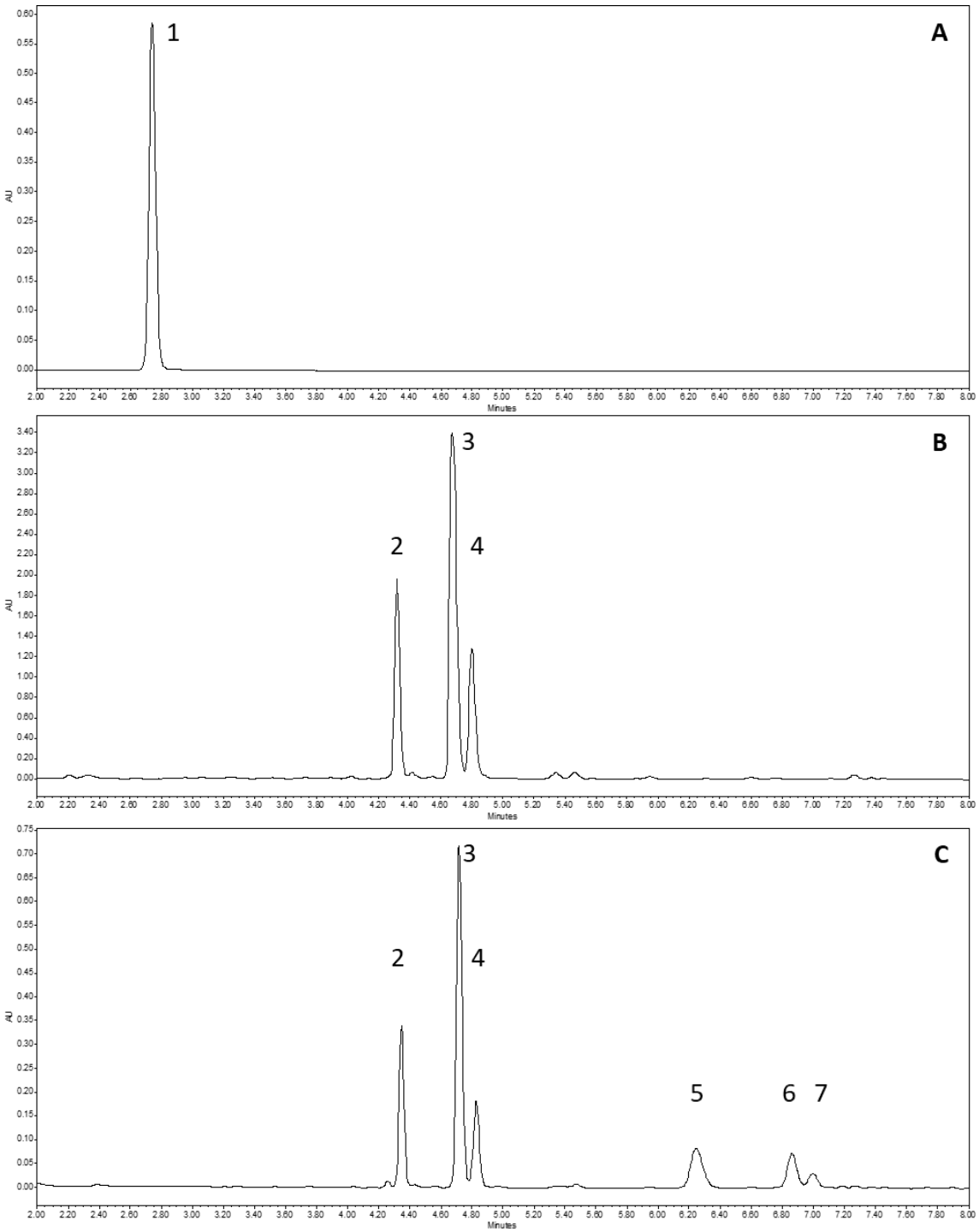

Figure 2. UHPLC-UV chromatograms (330 nm) of xanthohumol (**A**), mix of α-acids (**B**), and mix of αβ-acids (**C**). Identified compounds and their retention times (rt) were: xanthohumol (1) rt 2.78 min, co-humulone (2) rt 4.37 min, humulone (3) rt 4.75 min, ad-humulone (4) rt 4.86 min, co-lupulone (5) rt 6.31 min, lupulone (6) rt 6.92 min, ad-lupulone (7) rt 7.05 min.

3.2. In Vitro Anti-Oomycete Activity on Mycelial Growth and Spore Germination

3.2.1. Hop Cone Essential Oil Activity

Essential oil from hop cones was tested on the mycelial growth and germination of *P. infestans* and showed a slight anti-oomycete activity at the highest tested concentrations. Indeed, IC_{50} values were higher than 1000 mg·L^{-1} on mycelial growth and higher than 5000 mg·L^{-1} on spore germination (Table 4). Thus, the mycelium seemed more sensitive to the essential oil from the hop cones than the spores.

Table 4. Half maximal inhibitory concentration (IC_{50}) values of essential oil from hop cones on *P. infestans* mycelial growth and spore germination.

Mycelial Growth			**Spore Germination**		
IC_{50}	**Confidence Interval (95%)**		**IC_{50}**	**Confidence Interval (95%)**	
(mg·L^{-1})	2.5%	97.5%	(mg·L^{-1})	2.5%	97.5%
1 295	1 159	2 181	5 355	1 714	9 775

3.2.2. Extracts and Sub-Extracts Activity

The activity of hop crude hydro-ethanolic extracts (CHE) and DCM sub-extracts (DSE) was first evaluated on mycelial growth and spore germination of *P. infestans*.

For assays on solid medium, all tested extracts inhibited partially or completely the mycelial growth at the range of tested concentrations (Figure 3A). The extracts of leaves, both CHE and DSE, appeared to have a better anti-oomycete activity than the other hop parts, with an IC_{50} of 37.8 mg·L^{-1} and 13.4 mg·L^{-1} respectively (Figure 3A,B). Interestingly, the CHEs of cones, stems and rhizomes showed the same activity against *P. infestans* and were significantly less active than CHE of leaves. Similarly, the DSE of leaves had an IC_{50} statistically lower than other hop parts. For the same hop part, a different activity between types of extract was only observed for leaves, with the DSE being more active than the CHE. On the contrary, there was no significant difference between CHE and DSE activity of cones, stems and rhizomes.

All the tested extracts showed an antifungal activity on *P. infestans* spore germination in a glucose peptone medium (Figure 4A). However, extracts of leaves seemed to be more effective than other hop extracts (Figure 4B). Indeed, even though the CHE of leaves was not statistically different from CHE of cones and rhizomes, the DSE of leaves was the most active extract with an IC_{50} of 33.7 mg·L^{-1} . These results also showed that the DSE of leaves was more active than the CHE of leaves (Figure 4A). Unlike extracts of leaves, there was no difference of activity between the CHE and the DSE for cones, stems and rhizomes, nor between these three hop parts.

3.2.3. Prenylated Phenolic Compound Activity

Purified compounds of cones were tested on mycelial growth. Both xanthohumol and the bitter acid mix showed anti-oomycete activity (Figure 5A). The α-acids mix was significantly more active than xanthohumol, with an IC_{50} of 29.1 mg·L^{-1}. Xanthohumol showed the highest IC_{50} (191.2 mg·L^{-1}). It should be noted that mycelium was not completely inhibited by this compound, with a maximal inhibition of 80% recorded at 500 mg·L^{-1}. Copper sulfate, used as a reference, was more active than hop compounds with an IC_{50} of 2.2 mg·L^{-1}.

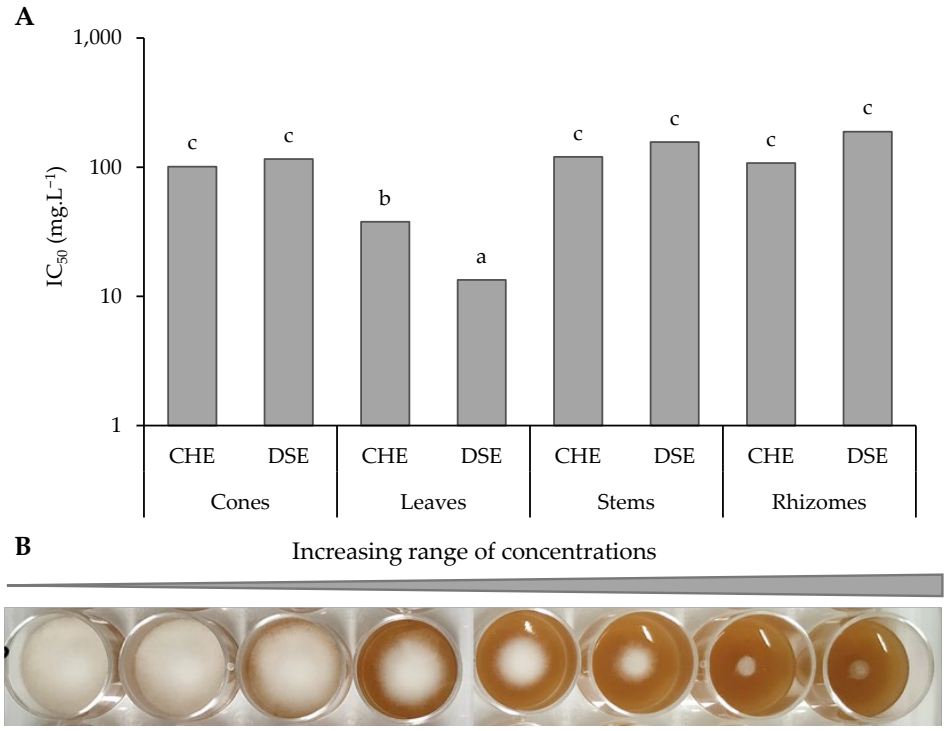

Figure 3. Anti-oomycete activity of hop extracts and sub-extracts on *P. infestans*_mycelial growth. (**A**) Half maximal inhibitory concentration (IC_{50}) values. (**B**) Inhibition of mycelium growth on V8 medium by a range of concentrations (0 to 250 mg·L^{-1}) of DSE of leaves after 10 days. Different letters represent significant differences.

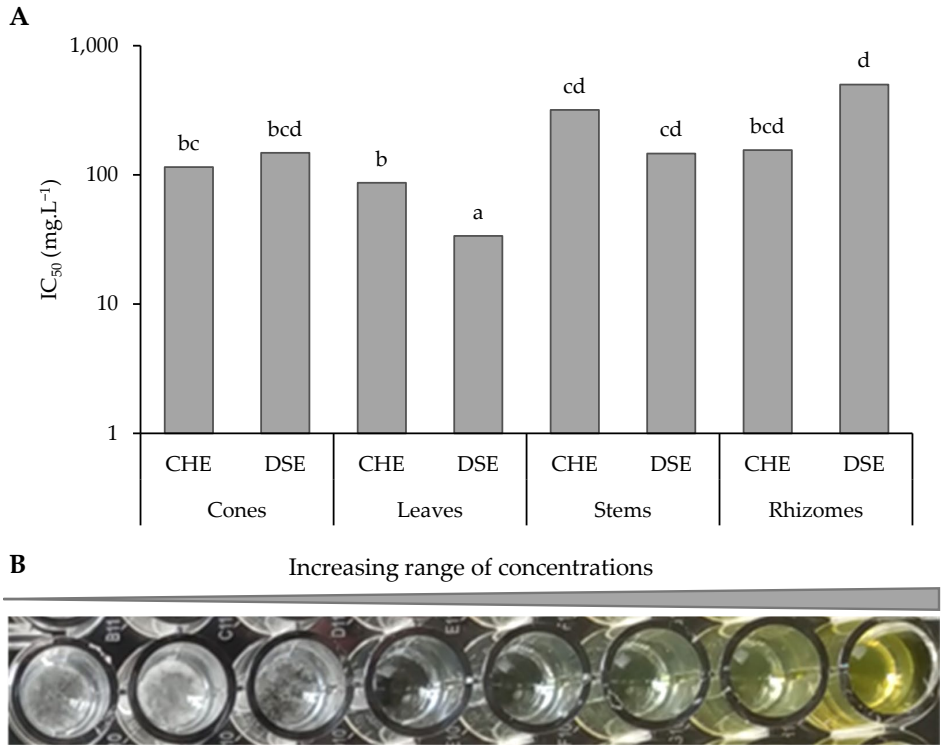

Figure 4. Anti-oomycete activity of hop extracts and sub-extracts on *P.infestans*_germination. (**A**) Half maximal inhibitory concentration (IC_{50}) values. (**B**) Inhibitory activity of DSE of leaves (from 0 to 1000 mg·L^{-1}) after six-day incubation.Different letters represent significant differences.

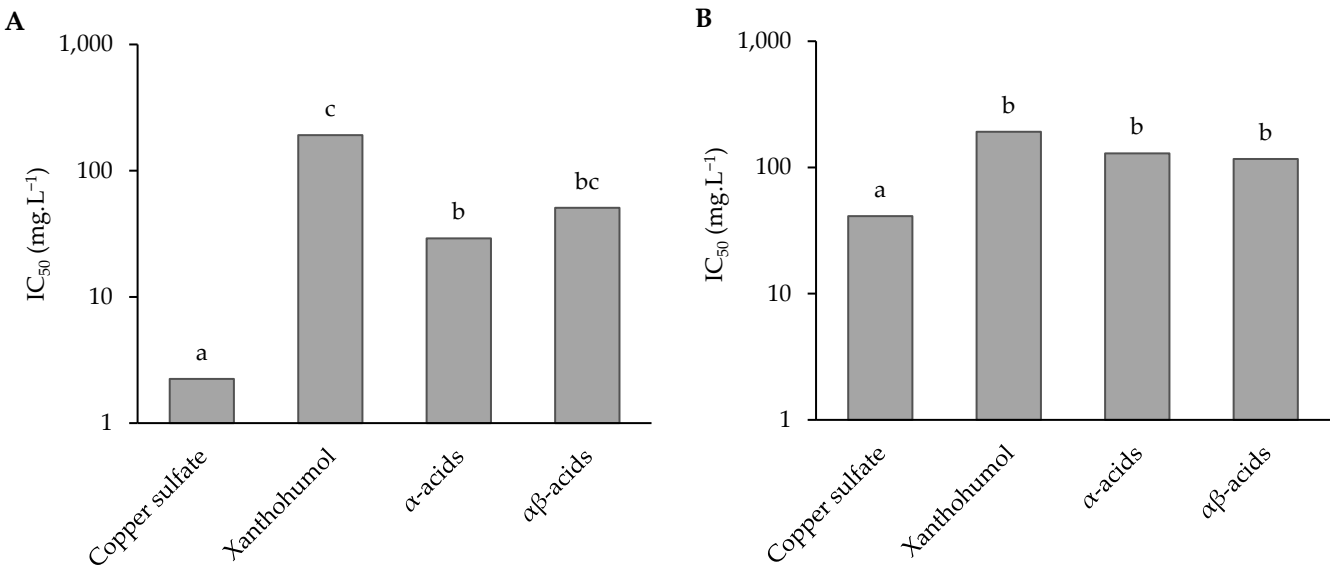

Figure 5. Half maximal inhibitory concentration (IC_{50}) values of hop main compounds and copper sulfate on *P. infestans* mycelial growth (**A**) and germination (**B**). Different letters represent significant differences.

Interestingly, these differences between bitter acids and xanthohumol were observed for *P. infestans* mycelial growth but not for its germination in the microplate. Indeed, xanthohumol, the α-acids mix, and the αβ-acids mix had a relatively similar activity with IC_{50} values of 191.5, 129.3 and 116.8 mg·L^{-1} respectively (Figure 5B). They were also less active than copper sulfate (IC_{50} of 41.2 mg·L^{-1}).

3.2.4. Morphological Modifications of the Mycelium

Observations for each extract and their metabolites are summarized in Table 5 and changes of the mycelium morphology under different conditions are illustrated in Figure 6.

Table 5. Optical observations of *P. infestans* mycelium morphology under different hop part extracts, metabolites, and copper sulfate treatments.

		Branched Mycelium	Lysed Mycelium
Untreated		-	-
Cones	CHE	+	+
	DSE	+	+
Leaves	CHE	+	+
	DSE	0	0
Stems	CHE	-	+
	DSE	-	+
Rhizomes	CHE	+	+
	DSE	-	+
Copper sulfate		+	+
Purified compounds	Xanthohumol	+	+
	α-acids	+	+
	αβ-acids	+	+

+ : presence of branched mycelium/presence of lysed mycelium; - : absence of branched mycelium (= filamentous mycelium)/absence of lysed mycelium (= intact mycelium); 0 : only non-germinated sporangia or germinated sporangia reforming sporangia.

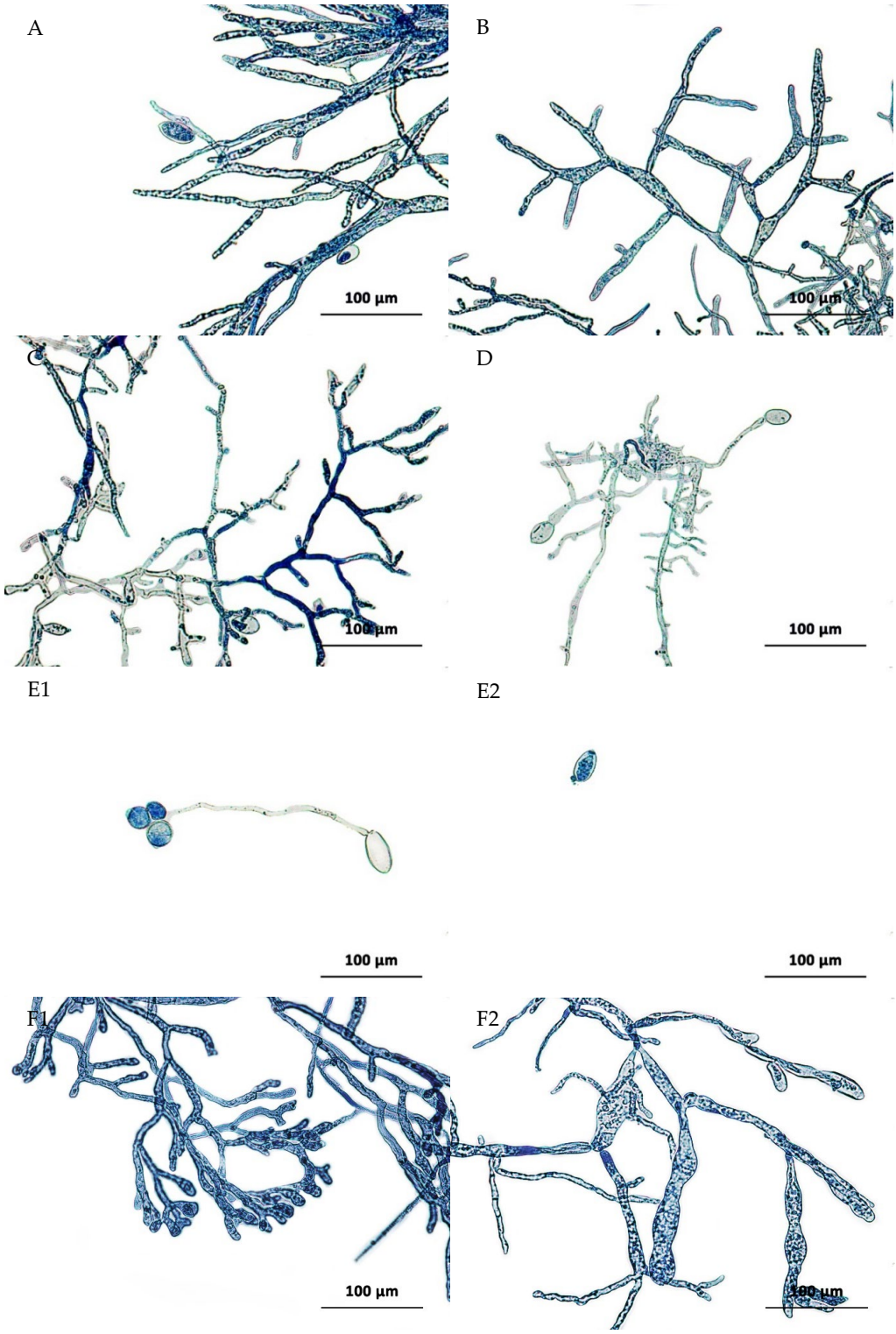

Figure 6. Optical microscopy images taken after 7 days of incubation of *P. infestans* in glucose peptone medium mixed with hop constituents and copper at concentrations close to their IC_{50}. (**A**) Untreated control; (**B**) Illustration of branched mycelium; (**C**) Illustration of branched and partially lysed mycelium; (**D**) Illustration of lysed mycelium; (**E**) Germinated sporangia reforming sporangia (1) and non-germinated sporangia (2) under the DSE of leaves treatment; (**F**) Xanthohumol treatment leading to branched tips (1) and thick mycelium (2).

First, the mycelium from the untreated control was observed and appeared to be well developed with filamentous hyphae (Figure 6A). Under hop treatments, important morphological changes were noticed with all extracts of the four hop parts at concentrations around IC_{50} values (Table 5). Indeed, the mycelium looked more branched for extracts of cones (CHE and DSE), as well as the CHE of leaves and rhizomes (Figure 6B,C). Moreover, the mycelium seemed partially or entirely lysed for all hop extracts (Figure 6C,D). Interestingly, mycelium under the DSE of leaves treatment was quite different from other treatments. Most of the sporangia did not germinate (Figure 6E2), but some germinated with a small hypha, directly reforming sporangia (Figure 6E1).

Microscopical observations were also performed for hop compounds and copper sulfate. Under copper treatment, the mycelium seemed more branched than the control one and started to have few lysed hyphae. Observations of the mycelium treated with the α-acids mix and αβ-acids mix were similar to hop extract treatments (Table 5). However, the treatment with xanthohumol alone seemed to have a different impact on mycelium morphology. Interestingly, the mycelium looked much more branched on the tips (Figure 6F1) and thicker (Figure 6F2) than the control one. The mycelium treated with xanthohumol also looked partially lysed.

4. Discussion

The present study was focused on anti-oomycete activity of various hop extracts and some of their specialized metabolites on *P. infestans*. The originality lies in the attempt for adding value to unexploited hop parts, in particular leaves, that are considered as by-products by the brewing industry. Hop is a dioecious plant species, and only the female inflorescences are used because they contribute to the bitterness, aromaticity and conservation of beer. To a lesser extent, the cones are also present on the market of food supplements [3]. However, in order to develop circular economy, the emphasis could be placed on promoting the whole plant and seeking outlets for the use of agricultural by-products [53]. Hop leaves are mainly recycled by composting [31].

The anti-oomycete activity was evaluated for both hop cone essential oil and extracts from the different parts of the plant (crude hydro-ethanolic extracts and apolar sub-extracts). The composition of the essential oil from cones presented in this study was consistent with Langezaal et al. [23], as myrcene, β-caryophyllene and α-humulene were the three major terpenoids identified. In addition, this study confirmed the results presented by Zanoli and Zavatti [8], which showed that bitter acids are the main compounds of cone extracts, where α-acids (co-humulone, humulone and ad-humulone) and β-acids (co-lupulone, lupulone and ad-lupulone) form a complex mix. Flavonoids are also present in these extracts, especially xanthohumol, which is the main prenylated chalcone. The quantification of these main phenolic compounds in the CHE of cones was consistent with data from Bocquet et al. [18] for the Nugget cultivar. The liquid/liquid extraction by dichloromethane allowed us to concentrate further these prenylated phenolic compounds, in particular humulone, and to eliminate the most polar molecules, such as flavonoid glycosides [3], to compare the anti-oomycete activity with the crude extract.

The metabolome of hop agricultural by-products, including the leaves, stems and rhizomes, was barely studied [18,54,55]. Bocquet et al. [18] already reported that leaves produced bitter acids and xanthohumol, with lupulone being the most abundant compound, which was confirmed in this study. However, their levels are lower than those in cones. Lupulone is about 10 times less concentrated in leaves than in cones. DSE and CHE of leaves contain similar amounts of these phenolic compounds. The presence of co-humulone and co-lupulone in leaves seems to depend on the hop cultivar, as Mishra et al. [56] noticed their absence in Osvald's 72 cultivar. Moreover, xanthohumol and humulone have already been identified in the CHE of stems, but for the first time xanthohumol, α-acids (co-humulone, humulone and ad-humulone) and lupulone were quantified in DSE. Even though, neither xanthohumol nor bitter acids were present in the CHE of rhizomes, xanthohumol and α-acids were quantified in the DSE.

The antifungal activities of hop extracts and some of their metabolites were mostly studied on human pathogens [1,4,5] and more recently on plant or post-harvest pathogens [6, 22,28,57]. The anti-oomycete activity of hop extracts from four parts (rhizomes, stems, leaves and cones) against *Phytophthora infestans* was evaluated for the first time. The results revealed the potential of female cones and agricultural by-products to inhibit mycelial growth and spore germination despite differences in extract composition. Both the CHE and DSE of cones revealed close and interesting activity on *P. infestans* that could be linked to their relatively similar compositions in xanthohumol and bitter acids. Antifungal activity has already been attributed to hop cones. Arruda et al. [28] and Nionelli et al. [26] demonstrated the potential activity of cones on several post-harvest pathogens such as *Byssochlamys nivea* and *Penicillium* spp. A crude hydro-ethanolic extract of cones also appeared to be the most active extract of the plant on the phytopathogenic fungus *Zymoseptoria tritici*. Indeed, Bocquet et al. [6] showed that the crude extracts of leaves, stems, and rhizomes did not really inhibit the mycelial growth of the fungus, whereas the crude extract of cones was the most active with an IC_{50} of 730 mg·L^{-1}. In that study, the essential oil from cones was even more active with an IC_{50} of 360 mg·L^{-1}. Interestingly, opposite results were observed on *P. infestans* as the essential oil from cones was only slightly active. Through this study, the CHE of cones seemed to be more active on the *P. infestans* oomycete than on *Z. tritici* fungus. Moreover, unlike Bocquet et al. [6], the CHE of leaves appeared to be the most active CHE on the spore germination and mycelial growth of *P. infestans*, with an IC_{50} of 37.8 mg·L^{-1} and 86.8 mg·L^{-1} respectively. The CHE of stems and rhizomes had anti-oomycete activity close to the CHE of cones. A recent study also showed the potential of hop stem and root extracts against *Verticillium nonalfalfae* mycelial growth with an IC_{50} between 130 and 212 mg·L^{-1} for stems and between 1172 and 1804 mg·L^{-1} for roots [57]. These results are particularly relevant with a view of finding a particular application of agricultural by-products of hop production.

Many plant extracts or essential oils already appeared to be active in vitro on mycelial growth [41,42,58] and spore germination of *P. infestans* [44–46] but in most of the cases high concentrations were tested. However, Abdelgaleil et al. [59] evaluated several extracts of *Curcuma longa* L. rhizomes on mycelial growth and obtained an EC_{50} equal to 159.8 mg·L^{-1} for the most active extract. Concerning germination, Rogozhin et al. [60] demonstrated that the most active tested product was a mixture of peptides from three plants (*Chelidonium majus*, *Inula helertium*, and *Equisetum arvense*) with an IC_{50} of 250 mg·L^{-1}. Thus, the results in this study showed that hop extracts have an interesting potential to control *P. infestans* in in vitro conditions, with an IC_{50} ranging from 13.4 to 188.5 mg·L^{-1} for mycelial growth and from 33.7 to 500 mg·L^{-1} for spore germination.

This study also aimed to determine the anti-oomycete activity of well-known hop phenolic compounds, xanthohumol and bitter acids. Purified xanthohumol, α-acids mix, and αβ-acids mix were active on *P. infestans*. α-acids were more active than xanthohumol, especially on oomycete mycelial growth, with an IC_{50} equal to 29.1 mg·L^{-1}. However, hop compounds remained less active than copper sulfate, a mineral fungicide causing environmental problems. Besides its antifungal activity against human pathogenic fungi [24], co-humulone demonstrated interesting antifungal activity against the phytopathogenic fungus *Z. tritici* [6]. Biofungicide properties of xanthohumol were only demonstrated on human pathogenic fungi [1] but not on phytopathogenic fungi yet. However, its close analogue, desmethylxanthohumol, has shown a weak antifungal activity against *Z. tritici*.

The active identified compounds, xanthohumol and bitter acids, are mainly present in hop cones and this could not explain the activity of other parts of the plant as they were only barely detected in some extracts. Thus, other compounds not yet identified might be behind this anti-oomycete activity. For now, there is no clear link between the anti-oomycete activity of extracts and the content of main metabolites of cones.

Even though the main prenylated phenolic compounds of hop cones are not responsible for the activity of all parts, every extract showed close morphological changes of the mycelium grown in liquid medium. A branched and/or lysed mycelium was observed

under the optical microscope. Morphological damages on *P. infestans* were already observed under several treatments with compounds from microbial origin [61–63] but also with botanicals [41,60,64]. The mode of action was not determined here, but according to the Fungicide Resistance Action Committee (FRAC), plant extracts could have an impact on cell walls and membranes (FRAC BM01). Thus, lyses and ramification of the mycelium observed under hop treatments could be linked to the loss of cell wall integrity or membrane permeability.

5. Conclusions

For the purpose of seeking alternatives to conventional treatments to manage diseases caused by *P. infestans*, the anti-oomycete potential of essential oil from hop cones as well as crude hydro-ethanolic extracts and apolar sub-extracts of different parts of hop plant was evaluated. The essential oil from hop cones was not very active. By contrast, the crude and apolar extracts of cones, leaves, stems and rhizomes displayed an in vitro activity on both mycelial growth and spore germination of *P. infestans.* Some prenylated phenolic compounds were also tested and showed a moderate anti-oomycete activity. α-acids were more active than xanthohumol, especially on oomycete mycelial growth. The activity of these specialized metabolites could partly explain the activity of cone extracts, but is not sufficient to elucidate the full activity of the other parts of the plant where these compounds are less concentrated. Further investigations will be required to identify other active compounds and to fully understand the anti-oomycete activity of these hop parts. In addition, many hop extracts tested also induced changes in mycelium morphology. In conclusion, leaves seem to be the most promising hop part against this oomycete, which makes this abundant agricultural by-product particularly interesting for pest management.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12112826/s1, Figure S1: GC-MS chromatogram of the commercial essential oil of hop cones. Main compounds were identified as myrcene (1), trans-caryophyllene (2), and α-humulene (3); Figure S2: Content (in $\mu g \cdot mg^{-1}$) of prenylated chalcones and acylphloroglucinols in (A) Crude hydro-ethanolic extracts (CHE) and (B) DCM sub-extracts (DSE) of different hop parts (n = 3, mean ± SD).

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