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Supercritical fluid-assisted extraction of hop cone residue from craft breweries

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ABSTRACT

This study aimed to use supercritical fluid-assisted extraction in hop cone residue from craft breweries and define the best extraction parameters. Yield, compounds extracted, in vitro chemical and cell-based antioxidant activities, and antimicrobial and cytotoxic potentials were evaluated. The variables studied were temperature (40–60 °C), pressure (10–20 MPa), and ethanol percentage as co-solvent (5–10%) using a Taguchi experimental design (11 runs). The extracts showed inhibitory action towards Gram-positive and Gram-negative bacteria, mainly *Proteus mirabilis* and *Enterococcus faecalis* (MICs = 5–10 mg/mL). Furthermore, up to the maximum tested concentration (400 μg/mL), the extracts showed no hepatotoxicity on a healthy porcine liver cell line (PLP2), indicating safety for food and pharmacological applications. Lower temperatures (40 °C), pressures (10 MPa), and ethanol percentages (5%) resulted in higher concentrations of some compounds (alpha-copaene, γ-elemene, γ-muurolene, α-muurolene, caryophyllene oxide) and higher antioxidant activity in cell-based assays, but lower yields. At the same time, lower temperatures (40 °C) and pressures (10 MPa) but higher ethanol percentages (10%) resulted in higher concentrations of other compounds (nerolidyl acetate, linalyl isobutyrate, α-acorenol, and geranyl isovalerate) and increased antioxidant activity in chemical systems. Our results support that the hop cone residue can be used to obtain supercritical fluid-assisted extracts with biological potentialities that might virtually be upcycled into multifunctional ingredients for the food and pharmacological industries. Using 40 °C, 10 MPa, and 5 or 10% ethanol percentage as process parameters is advisable.

1. Introduction

Hops (*Humulus lupulus* L.) is a dioecious, climbing, and perennial plant that belongs to the *Cannabaceae* family. Its cultivation generally occurs in temperate regions around the world. The female plant produces an inflorescence called a cone, formed by bracts and containing the lupulin gland. This gland can synthesize secondary compounds such as essential oil, bitter acids, and polyphenols with bioactive properties [1–3].

Traditional medicine has used the hop cone for many years to treat gastric diseases, fever, insomnia, anxiety, and stress [4]. In addition, in the literature, it is possible to observe studies reporting antioxidant [5], antimicrobial [6], and antifungal potential [7] for the hop compounds.

The hop cone is overvalued in brewing because it contributes to bitterness, aromatic notes, foam stability, and microbiological control in beer. Currently, beer is the most consumed alcoholic beverage in the

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world [8,9]. Its production chain generates a large volume of solid waste. To produce 1 m^3 of beer, around 51.2 kg of solid by-products are generated, representing a great environmental impact. In this way, these residues, including hop cones, can have compounds with interesting nutritional values [10,11].

The most used methodologies for obtaining oils and extracts from residues are steam distillation and the utilization of organic solvents. The distillation process uses high temperatures, which can reduce the final product's quality and degrade thermolabile substances. The use of solvents requires separation processes for solvent removal, which mostly have toxic characteristics. Furthermore, important compounds may be lost during this separation process [12].

On the other hand, emerging methodologies, such as supercritical fluid-assisted extraction, may be used as alternatives to traditional processes. This technique uses fluids at temperatures and pressure above their critical point, reaching their supercritical state. In this state, solvents can easily diffuse through the plant matrix, and the target compounds can be extracted more efficiently. In addition, milder temperatures are used, and it is possible to prioritize the extraction of a given compound by adjusting the temperature, pressure, and solvent [13,14].

Carbon dioxide (CO_2) is the main solvent used in this methodology because it has a relatively low cost, is non-toxic, non-flammable, does not form chemical residues in the extract, and has a non-polar characteristic. Therefore, it can extract the soft resin from the hop matrix. The resin substances are responsible for the aromatic notes of hop. Furthermore, adding ethanol to this process can lead to greater heterogeneity of compounds and polyphenols in the extract due to its polar and non-toxic characteristics [15–17].

Previous literature has explored the characterization of hop cone extracts utilizing traditional extraction methods [18,19]. Although certain investigations have employed supercritical fluids to extract hop cones and leaves [20–26], no studies have yet examined the application of supercritical fluid extraction for hop cone residues derived from craft breweries. Still, no in vitro antioxidant potential was evaluated. Therefore, this study aimed to use supercritical fluid-assisted extraction in hop cone residue from craft breweries and define the best extraction parameters. Yield, compounds extracted (analyzed by chromatography coupled with mass spectrometry (GC-MS)), in vitro chemical and cell-based antioxidant activities, and antimicrobial and cytotoxic potential were evaluated.

2. Material and methods

2.1. Sample

The hop cone residue of the Centennial variety (Yakima Chief Hops®), used by an artisanal brewery located in the city of Maringá-PR, Brazil, was provided for the study. This residue was lyophilized at − 86 °C (LIOTOP - L101, Liobras, São Carlos, Brazil), crushed in a knife mill (Solab SL-30; Solab, Piracicaba, Brazil), vacuum-packed in packages protected from light, and stored at a temperature of – 24 °C until the extraction process. The fraction of the ground sample that passed through the 16 Tyler mesh (mean particle diameter: ~ 1.19 mm) was used for oil extraction.

2.2. Extraction assisted by supercritical fluid

The extraction of hop residue was performed using the experimental apparatus presented in Fig. 1. This apparatus consists of two high-pressure syringe pumps (Teledyne ISCO, Model 500D, Lincoln, Nebraska, USA) connected to a controller (Teledyne ISCO, model 500D, Lincoln, Nebraska, USA), an stainless steel extractor vessel (1.95 cm of diameter and 19.4 cm of height) with a capacity of 58 cm^3 and two thermostatic baths (Julabo, Model F25-ME, Seelbach, Baden-Württemberg, Germany) to cool the syringe pumps and the extraction vessel

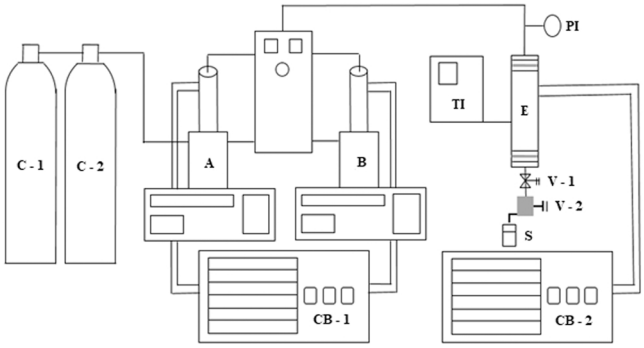

Fig. 1. Schematic diagram of the extraction equipment: C-1 carbon dioxide cylinder; C-2 ethanol vessel; CB-1 and CB-2 circulation baths; A carbon dioxide syringe pump; B ethanol syringe pump; PI pressure indicator; TI temperature indicator; E extractor; V-1 needle valve; V-2 micro-metering needle valve; S sample collection tube.

(Fisatom, model 806, São Paulo, São Paulo, Brazil). The high-pressure syringe pump (Bench Top, Dual Pump series) used in the extractions controls specified pressure, total volumetric flow rate, each solvent's percentual volumetric flow rate, and total volume reservoir conditions. It can operate at either constant pressure or a constant flow rate.

The extractor vessel has two 200-mesh wire filters (one at each end), and the vessel was loaded with the hop residue. The system was then heated and pressurized to the desired temperature and pressure. The static extraction period was performed with the solvent maintained in contact with the hop residue for 20 min. Throughout the dynamic extraction process, conducted at a steadfast solvent flow rate of 2.0 ± 0.1 mL/min, ascertained under the conditions of the syringe pump apparatus, the hop extract was conserved in previously weighed, light-protective (amber) containers, possessing a predetermined mass. For each extract, ~10 g of hop residue was used, and an extraction time of 60 min. The extraction conditions of temperature (°C), pressure (MPa), and volumetric flow rate percentage of ethanol (cosolvent/modifier) were adjusted and changed for each extract according to Table 1 (Taguchi design). The extract yield and its compounds were evaluated to determine the best extraction condition. The yield was calculated using the extract's mass and the residue's initial weight. To ensure the resultant extract is devoid of ethanol, samples were subjected to a forced ventilation oven (model 400/4ª, Ethik Technology, Vargem Grande Paulista, SP, Brazil) at 35 °C for 8 h.

The study followed the operational conditions of the extractions, such as temperature, pressure, flow rate, and CO_2 concentration, using an ethanol + CO_2 mixture as reported in Table 2 by previous studies. The temperature, pressure, and percentage of ethanol ranges were chosen to keep the solvent close to its critical point and prevent solute thermal damage. Furthermore, it has been reported that pressures higher than 20 MPa have no significant impact on hop extract yield and composition [12].

Table 1
Extraction conditions and extract yield.

Run	Temperature (°C)	Pressure (MPa)	% (v:v) solvent (ethanol)	Yield %
E1	40	10	5	7.17
E2	60	10	5	13.35
E3	40	20	5	19.45
E4	60	20	5	24.7
E5	40	10	10	13.32
E6	60	10	10	12.04
E7	40	20	10	24.20
E8	60	20	10	14.22
E9	50	15	7.5	16.41
E10	50	15	7.5	11.86
E11	50	15	7.5	19.22

Table 2
Extraction conditions report in literature.

Vegetal matrix	Temperature (°C)	Pressure (MPa)	Other information's	Ref.
Hop pellet of hop (*Humulus lupulus L*)	35, 45 and 55	10 and 20	CO_2 solvent, $3.25 \bullet 10^{-5}$ kg/s mass flow, 55 °C and 20 MPa optimal operating.	[12]
Flowers of hop (*Humulus lupulus L*)	40, 60, 80	15, 20, and 25	CO_2 solvent, 2.0 mL/min volumetric flow, ethanol; ethanol or ethy acetate modifier, 60 min static extraction period	[20]
Cones and leaves of hop (*Humulus lupulus L*)	40	20	CO_2 solvent, 40 g sample weight,1 L extraction vessel.	[22]
Cones of hop (*Humulus lupulus L*)	50	30	CO_2 solvent, 1.8–32.3 nm size particles.	[23]
Cones of hop (*Humulus lupulus L*)	20, 40, 60 and 80	5, 10, and 15	CO_2 solvent, 40 °C and 10 MPa optimal operating.	[24]
Cones flowers of hop (*Humulus lupulus L*)	50	35	CO_2 solvent, 7 g sample weight, 290 mL extraction vessel, 10 min static extraction period.	[25]

2.3. Chromatographic characterization

The extracts were analyzed on a gas chromatograph (Agilent 7890B) coupled to a mass spectrometer (Agilent 5977 A MSD), operating with an electron source with an ionization energy of 70 eV. An HP-5MS IU capillary column (30 m x 0.25 mm × 0.250 mm) filled with a stationary phase of 5% phenyl and 95% dimethyl polysiloxane was used. The injected volume of samples, properly diluted, was 2 μL, under the oven programming conditions: initial temperature of 50 °C and maintained for 3 min followed by heating at 3 °C/min until the final temperature of 300 °C, remaining for 10 min. Sample injection was performed in split mode at a 1:20 ratio with a constant flow of 1.0 mL/min of helium as a carrier gas, with the injector temperature maintained at 250 °C and the transfer line at 290 °C. In the mass detector, the ionization chamber temperature was 230 °C, and the quadrupole temperature was 150 °C. In the mass spectrometer, the EM detection system was used in "scan" mode, operating in the mass/charge ratio range (*m/z*) of 40–600, with a "solvent delay" of 3 min

The compounds were identified by comparing their mass spectra with the mass spectra of the NIST 11.0 library and by comparing their retention indexes (RI) obtained by a homologous series of the n-alkane standard, C7 to C40. The concentrations were obtained by analyzing each chromatographic peak's relative area compared to the octadecane (Sigma-Aldrich) internal standard's (2,5 μg mL^{-1}) peak area [27].

2.4. Antioxidant activity

To investigate the antioxidant potential of the hop cone residue extracts, five distinct in vitro techniques were employed: two cell-based methods (1) oxidative hemolysis inhibition assay (OxHLIA) and (2) the inhibition of the production of thiobarbituric acid reactive substances (TBARS); in addition to three chemical assays (3) reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH); (4) reduction of the 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) cation and (5) reduction power of the ferric ion (FRAP).

2.4.1. Oxidative hemolysis inhibition assay (OxHLIA)

The analysis of the OxHLIA method was performed using an erythrocyte solution (2.8%, v/v; 200 μL) prepared with a phosphate-buffered saline solution (PBS, pH 7.4), which was homogenized with 400 μL of extract solution (2.03–130 μg/mL in PBS) and Trolox (positive control; 7.81–125 μ). These mixtures were pre-incubated for 10 min at 37 °C with continuous agitation. Then, 200 μL of 2,2′-azobis(2-methyl-propionamidine) dihydrochloride (AAPH; 160 mM in PBS) was added, and the optical density was kinetically measured at 690 nm using an ELx800 microplate reader (Bio -Tek Instruments, Winooski, VT, USA) to hemolysis. The IC_{50} values (μg/mL) for 60 min (Δt) were obtained by correlating the extract concentration to the Δt values (min), which resulted from half the hemolysis time (Ht_{50} values) obtained from the hemolytic curves of each concentration of extract minus the Ht_{50} value of the PBS control [28].

2.4.2. TBARS test

Analysis of the thiobarbituric acid reactive substances (TBARS) method measures the ability to inhibit oxidation in a pre-incubation phase. For this method, 200 μL of sample solution (extracts from each sample) were diluted, then mixed with 100 μL of $FeSO_4$ (10 μM) and 100 μL of ascorbic acid (0.1 mM) in an Eppendorf reaction tube (2 mL). This mixture was pre-incubated for 1 h at 37 °C. Subsequently, the reaction mixture was completed with 500 μL of trichloroacetic acid (28% w/v) and 380 μL of thiobarbituric acid (TBA, 2% w/v). This resulting solution was heated for 20 min at 80 °C. Finally, the reaction tubes were centrifuged at 3000g for 10 min, and to quantify the malondialdehyde (MDA)-TBA complex, the absorbance of the supernatant was read at 532 nm. The results were expressed as IC50 values (μg/mL) [28].

2.4.3. DPPH radical scavenging activity assay

The analysis of antioxidant activity by the DPPH (2,2-Diphenyl-1-picrylhydrazyl) method followed the protocol described by Rufino et al. [29] and Ma et al. [30]. In tubes protected from light, 25 μL of the diluted extract (1 mg/mL) and 2 mL of the 6.25×10^5 mol/L DPPH solution were added and kept for 30 min. Subsequently, the reading was performed in a spectrophotometer (Agilent Cary 60 UV-Vis) at 517 nm. An analytical standard curve with Trolox solution [(±)− 6-hydroxy-2, 5,7,8-tetramethyl chroman-2-acid carboxylic] was constructed (100–2000 μmol/L, $R^2 = 0.9918$). The results were expressed in μmol Trolox/mg dry weight (DP).

2.4.4. ABTS radical scavenging activity assay

Antioxidant activity by the ABTS method (2,2′-azino-bis-(−3-ethyl-benzothiazoline-6-sulfonic acid), diammonium salt, ~98%) was determined according to Rufino et al. [31]. In light-protected tubes, 30 μL of the extract (1 mg/mL) and 3 mL of the ABTS+ solution was placed in the dark at room temperature for 6 min, and three measurements were taken for each sample and analyzed on average. The reading was performed in a spectrophotometer (Agilent Cary 60 UV-Vis) at 734 nm, and a standard curve with Trolox solution was constructed (50–2000 μmol/L, $R^2 = 0.9947$). Results were expressed in μmol Trolox/mg DW.

2.4.5. Reduction of iron (III) to iron(II) (FRAP)

The analysis of antioxidant activity using the FRAP iron reduction method started with the preparation of the FRAP reagent, adding 25 mL of acetate buffer (0.3 mol/L, pH 3.6), 2.5 mL of TPTZ solution (10 mmol/L [2,4,6-Tris(2-pyridyl)-S – triazine]) and 2.5 mL of ferric chloride hexahydrate solution ($FeCl_3$, 20 mmol/L). A mixture of 100 μL of the sample (1 mg/mL), 300 μL of distilled water, and 3 mL of FRAP reagent were added to test tubes protected from light, followed by homogenization, and kept in a water bath at 37 °C for 30 min. Then, the reading was performed in the spectrophotometer (Agilent Cary 60 UV-Vis) at 593 nm, and a standard curve with Trolox solution was constructed (100–700 μmol/L; $R^2 = 0.9954$). The results were expressed in μmol Trolox/mg DW. This assay was performed according to Rufino et al. [31] with modifications.

Hop extracts were diluted in methanol (1 mg of extract in 1 mL of methanol). This diluted extract was stored in an amber bottle to protect

against light and refrigerated until the DPPH, ABTS, and FRAP assays were performed.

2.4.6. Cytotoxic activity

The extracts obtained from the hop cone residue were suspended in water to obtain a stock solution of 8 mg/mL and diluted to obtain a range of five concentrations below the stock solution. The culture of non-tumor cells, called PLP_2, was created using a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established in preliminary tests. The sulforhodamine B assay was performed according to the previously described procedure [32]. Ellipticine was used as a positive control, and the results were expressed in GI_{50} values (μg/mL), corresponding to the sample concentration that provides 50% inhibition of cell growth.

2.5. Antimicrobial activity

The hop cone extracts were dissolved in water to obtain a stock solution of 20 mg/mL and were serially diluted to obtain the concentration ranges of 20–0.15 mg/mL. Different microorganisms were used, namely five Gram-negative bacterial strains: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Morganella morganii* (ATCC 8076), *Proteus mirabilis* (ATCC 29906), *Pseudomonas aeruginosa* (ATCC 27853) and three Gram-positive strains; *Enterococcus faecalis* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 25923). All microorganisms were purchased from Frilabo, Porto, Portugal. The minimum inhibitory concentrations (MIC) were determined by the serial microdilution method and by the *p*-iodonitrotetrazolium violet (INT) rapid colorimetric assay, following the protocol described by Soković et al. [33] and Soković and van Griensven [34]. MICs were delimited as the lowest concentration of extract that inhibits visible microbial growth (under a binocular microscope). Minimum bactericidal concentrations (MBC) were the lowest extract concentration required to inactivate the original inoculum. Five percent DMSO was used as a negative control. As positive controls, Streptomycin and Ampicillin were used. Results were given as MICs and MBCs in mg/mL.

2.6. Statistical analysis

All analyzes were performed in triplicates, and the data were analyzed using the Design of the Experiment (Taguchi analysis). The data were also analyzed using the Principal Component Analysis (PCA) with a matrix of 11 rows (11 extracts) and 22 columns (compounds, yield, and antioxidant activity). Hierarchical clustering analysis (HCA) was performed using the coordinates of the extracts in the first and second dimensions of the PCA map. Euclidean distances (dissimilarity), automatic truncation, and Ward's method (agglomeration method) were used. The dendrogram quality was evaluated using the cophenetic correlation coefficient. All statistical analyses were performed using XLSTAT 2022.2.1 (Adinsoft®, New York, USA).

3. Results and discussion

3.1. Extraction conditions and yield

The supercritical fluid extraction method proved to be a good process for the recovery of compounds from the residual cone of the brewery since the extracts presented remarkable yields (superior to 11.0%, Table 1, except E1). The pressure exerted a favorable influence on yield ($p = 0.03$), resulting in extracts E4 (24.70%), E7 (24.2%), and E3 (19.45%) exhibiting the most substantial yield values among the 11 extracts. Notably, these extracts employed elevated pressure values (20 MPa). Although temperature and solvent percentage had no significant impact ($p = 0.95$ and $p = 0.82$), E1 showed the lowest yield (7.17%), demonstrating that the mild conditions (40ºC, 10 MPa, 5% EtOH) resulted in lower extraction of compounds. However, the increased EtOH percentage for the same temperature and pressure conditions (E5) increased the yield. CO2 is considered the leading agent for extracting compounds from raw materials. Ethanol as a co-solvent or higher pressure can increase the solubility of some solutes in CO2, increasing the extraction rates and resulting in higher yields [20].

3.2. Chromatographic analysis

The main compounds of the terpene and bitter acid class identified by gas chromatography were compiled in Table 3 with their respective relative areas. A total of 16 compounds were detected, including terpenes, sesquiterpenes, oxygenated sesquiterpenes, and bitter acids. Caryophyllene, caryophyllene oxide, and humulene were identified in all extracts. At the same time, lupulone was present in almost all samples (except for sample E7). Lupulone and humulene are well-known compounds in the hop and present pharmacological and biological properties, such as cancer chemopreventive, sedative, antioxidant, and antimicrobial activities [19].

The increased EtOH percentage negatively impacted the contents of caryophyllene ($p = 0.04$) and caryophyllene oxide ($p = 0.02$), whereas temperature positively impacted the content of lupulone ($p = 0.05$). In this way, E1 - E4 extracts showed a higher concentration of caryophyllene and caryophyllene oxide, with predominance in E4 and E1, respectively. These extracts were obtained using the lowest ethanol percentage (5%). At the same time, E2, E4, E6, and E8 showed greater concentrations of lupulone, with predominance in E4. These extracts were obtained using the highest temperature (60 °C). Increases in temperatures may result in higher vapor pressure of solutes and CO2 diffusivity and increased solubility of some compounds in the CO2, leading to higher concentrations in the extract [20,26].

Some compounds were identified only in specific extracts, demonstrating that interactions among variables are important for compound extraction. For example, E1 presented alpha-copaene and γ-elemene, whereas E5 presented nerolidyl acetate, linalyl isobutyrate, α-acorenol, and geranyl isovalerate. These results indicate that moderate pressure and temperature conditions facilitated the preservation of elevated concentrations of the compounds above in the extracts, given that E1 and E5 were executed at 40 °C and 10 MPa. Moreover, the data underscore the significance of EtOH as a co-solvent, with distinct compounds being extracted contingent upon the concentration employed.

3.3. Antioxidant activity

The in vitro antioxidant potential of the hop residue extracts was evaluated by a set of chemical assays, namely DPPH, FRAP, and ABTS methodologies, which are often used to estimate the antioxidant activity of plant extracts [35]. The variables (temperature, pressure, and ethanol percentage) had no significant impact on the antioxidant activity ($p > 0.05$). However, E5 (DPPH: 1048.22 ± 7.89; FRAP: 746.00 ± 4.64 and ABTS: 1095.67 ± 13.30 μmol Trolox mg/DW), E6 (DPPH: 1084.55 ± 9.05; FRAP: 602.62 ± 7.96 and ABTS: 1134.63 ± 12.19 μmol Trolox mg/DW), E9 (DPPH: 1238.15 ± 6.09; FRAP: 550.36 ± 0.99 and ABTS: 1338.11 ± 10.38 μmol Trolox mg/DW) and E10 (DPPH: 1104.89 ± 2.50; FRAP: 568.88 ± 10.10 and ABTS: 1285.15 ± 15.27 μmol Trolox mg/DW) extracts exhibited better antioxidant activities for the three methodologies tested (Table 4). These results demonstrate that lower pressures concomitantly to medium or high ethanol percentages were important parameters to increase the antioxidant activity. In addition, the results of antioxidant activity corroborate those of chromatographic analysis.

It is important to highlight that the antioxidant potential herein reported for chemical assays is higher than those reported by previous studies [1,19,36,37], which indicates that the hop residue extracts present a chemical complexity of compounds reactive to free radicals. In this way, the hop cone, normally discarded, is a promising source for

Table 3
Compounds of the terpene and bitter acid class identified by GC-MS.

n	Compound	Relative area (%)										Class
		E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	
1	Alpha-Copaene	0.117%	-	-	-	-	-	-	-	-	-	-	H. S.
2	Caryophyllene	0.874%	0.873%	1.034	2.028	0.412	0.537	0.684	0.725	* 0.338% (0.321; 0.017)	* 0.461% (0.436; 0.025)	1.008	H. S.
3	Humulene	3.173%	1.767%	* 3.522% (3.109; 0.413)	* 7.324% (6.467; 0.857)	* 1.663% (1.324; 0.289)	* 1.776% (1.501; 0.275)	* 3.039% (2.505; 0.534)	* 2.17% (1.937; 0.233)	0.875	* 1.274% (1.069; 0.205)	* 3.523% (3.084; 0.439)	H.S.
4	γ-Muurolene	* 1.169% (0.377; 0.792)	-	0.458	-	0.271	-	-	-	-	-	-	H.S.
5	γ-Elemene	0.194%	-	-	-	-	-	-	-	-	-	-	H.S.
6	α-Muurolene	0.118%	-										H.S.
7	Caryophyllene oxide	* 5.471% (0.826; 0.496; 1.970; 2.050; 0.129)	2.211%	* 2.052% (0.570; 1.482)	1.627	* 0.782% (0.506; 0.276)	0.660	* 1.971% (0.521;1.450)	* 0.724% (0.559; 0.165)	* 1.085% (0.317; 0.768)	0.505	* 0.64% (0.495; 0.145)	S.O.
8	Ledene oxide-(II)	0.117%	-	-	-	-	-	-	-	-	-	-	H.S.
9	Trans-Z-α-Bisabolene epoxide	0.187%	0.956%	-	1.740	-	0.687	-	-	-	-	-	H.S.
10	Cis-Z-α-Bisabolene epoxide	0.073%	-	-	-	-	-	-	-	-	-	0.147	H.S.
11	Lupulon	* 26.475% (0.354; 13.064; 6.859; 1.024; 3.384; 1.057; 0.733)	* 22.756% (14.029; 8.727)	* 20.509% (11.647; 8.862)	* 30.504% (18.364; 12.140)	* 17.237% (9.256; 7.981)	* 29.598% (14.902; 14.696)	-	* 23.128% (11.444; 11.236; 0.448)	* 18.162% (9.275; 8.887)	* 17.306% (7.802; 6.686; 2.818)	* 15.43% (9.440; 5.990)	Á.A.
12	Cis-muurola-3,5-diene	-	0.286%	-	-	-	-	-	-	-	-	-	H.S.
13	Nerolidyl acetate	-	-	-	-	0.095	-	-	-	-	-	-	H.S.
14	Linalyl isobutyrate	-	-	-	-	0.069	-	-	-	-	-	-	H.S.
15	α-acorenol	-	-	-	-	0.236	-	-	-	-	-	-	H.S.
16	Geranyl isovalerate	-	-	-	-	0.044	-	-	-	-	-	-	H.S.

Table 4
In vitro antioxidant potential of the hop cone residue extracts.

Extract	*Cell-basead assays*				*Chemical assays*		
	OxHLIA EC_{50} (μg/mL) $\Delta t = 60$ min	Positive control Trolox (TE)	TBARS EC50 (mg/mL)	Positive control Trolox (TE)	DPPH (μmol ET Trolox mg/PS)	FRAP (μmol ET Trolox mg/DW)	ABTS (μmol ET Trolox mg/DW)
E1	37 ± 2	21.8 ± 0.2	2.59 ± 0.4	5.8 ± 0.6	658.89 ± 9.05	482.87 ± 4.59	997.22 ± 16.36
E2	97 ± 6	21.8 ± 0.2	6.10 ± 3.85	5.8 ± 0.6	572.78 ± 3.34	405.33 ± 3.38	815.17 ± 16.5
E3	55 ± 3	21.8 ± 0.2	2.1 ± 0.6	5.8 ± 0.6	524.44 ± 5.93	440.83 ± 2.00	824.70 ± 19.41
E4	190 ± 10	21.8 ± 0.2	1.71 ± 0.10	5.8 ± 0.6	259.44 ± 6.54	438.95 ± 3.05	633.83 ± 19.37
E5	142 ± 6	21.8 ± 0.2	10.8 ± 3.0	5.8 ± 0.6	1048.22 ± 7.89	746.00 ± 4.64	1095.67 ± 13.30
E6	111 ± 8	21.8 ± 0.2	2.0 ± 0.5	5.8 ± 0.6	1084.55 ± 9.05	602.62 ± 7.96	1134.63 ± 12.19
E7	304 ± 10	21.8 ± 0.2	0.45 ± 0.02	5.8 ± 0.6	330.55 ± 6.77	496.00 ± 5.41	747.52 ± 7.81
E8	273 ± 22	21.8 ± 0.2	0.3 ± 0.06	5.8 ± 0.6	903.89 ± 2.69	575.37 ± 6.13	1072.56 ± 17.82
E9	377 ± 11	21.8 ± 0.2	0.35 ± 0.06	5.8 ± 0.6	1238.15 ± 6.09	550.36 ± 0.99	1338.11 ± 10.38
E10	360 ± 14	21.8 ± 0.2	0.5 ± 0.16	5.8 ± 0.6	1104.89 ± 2.50	568.88 ± 10.10	1285.15 ± 15.27
E11	253 ± 9	21.8 ± 0.2	0.89 ± 0.05	5.8 ± 0.6	736.67 ± 8.66	504.95 ± 5.73	894.93 ± 10.28

compounds that significantly inhibit free radicals.

Likewise, the antioxidant potentials verified using cell-based assays are described in Table 4, with results expressed as EC_{50} (μg/mL). The extract that showed the best antihemolytic action by the OxHLIA assay was E1, with a value of 37 ± 2 (μg/mL). Yet the other extracts that showed better activities were E3 > E2 > E6 > E5 > E4. Capturing free

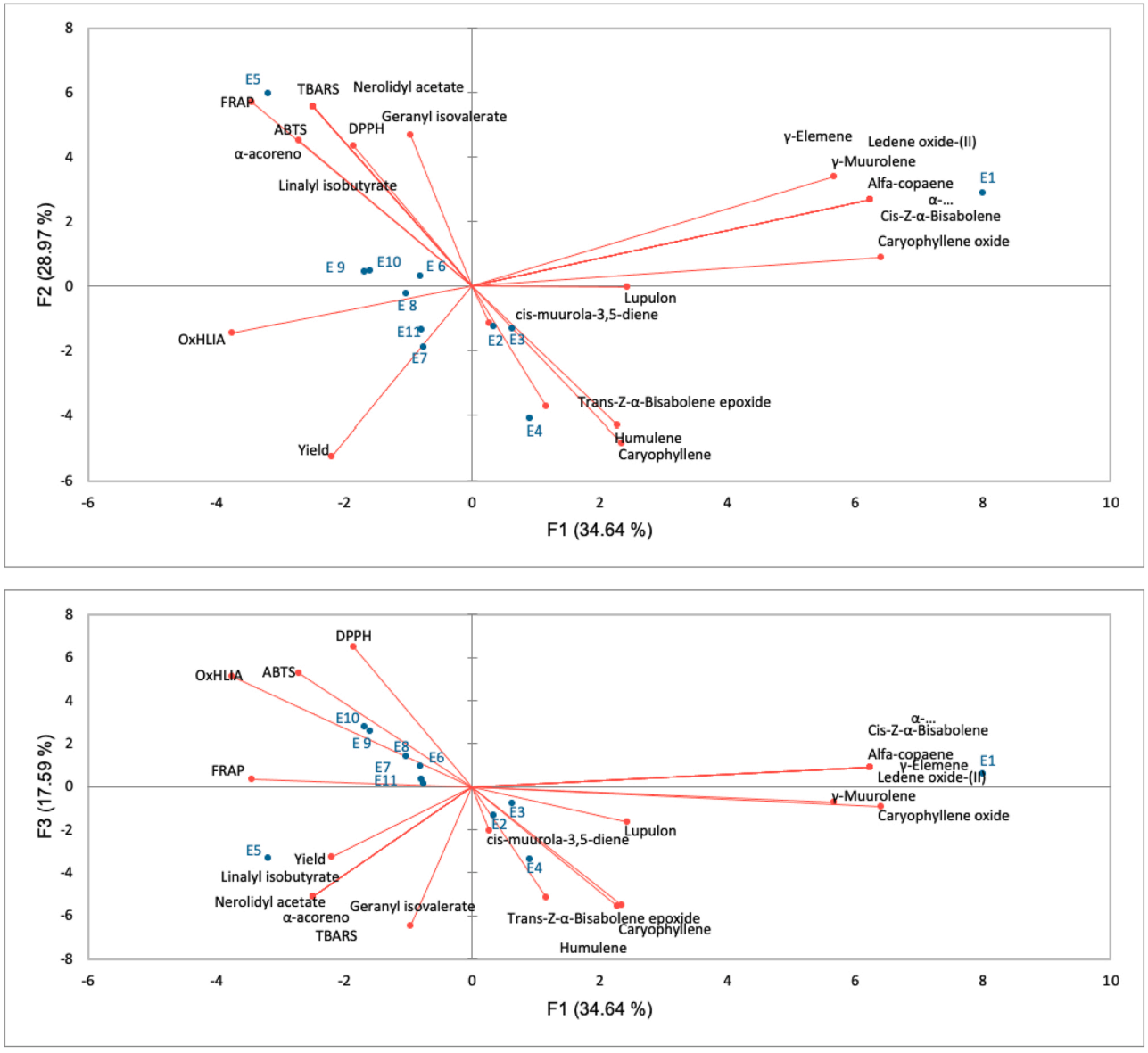

Fig. 2. PCA of the compounds, yield, and antioxidant activity.

radicals and altering cell membrane properties by some compounds can explain the possible mechanism in cell-related antioxidant activity. These compounds can permeate the membrane bilayer and lead to a decrease in fluidity and stability. With the reduction of membrane permeability, the diffusion of free radicals through it is limited, thus hindering the cell lysis process, and consequently, there is a cellular antioxidant potential [38]. And so far, this is the first result reported in the literature with this methodology to verify the biological potential of hop extracts.

The TBARS assay checks the ability of the extract to inhibit the formation of malondialdehyde and other end products of low molecular weight that are generated through *ex vivo* decomposition and lipid peroxidation. Porcine brain cells were employed because of their status as biological substrates abundant in polyunsaturated fatty acids, rendering them suitable models for investigating lipid peroxidation processes [39]. The extracts that showed the best EC_{50} values for this test were E8 > E9 > E7 > E10 > E11 > E4.

3.4. PCA map

The first three principal components (PC1, PC2, and PC3) explained 81.20% of the total variance of the data (PC1: 34.64%, PC2: 28.97%, PC3: 17.59%) (Fig. 2). PC1 separated E1-E4 (on the right side) from the other runs (left side). In this way, E1-E4, but mainly E1, were characterized by a higher concentration of alpha-copaene, γ-muurolene, γ-elemene, α-muurolene, caryophyllene oxide, ledene oxide-(II), and cis-Z-α-bisabolene.

PC2 separated E1 and E5 (above the axis) from the other extracts (intermediate and below the axis). In this way, E1 and, mainly E5, showed a higher concentration of nerolidyl acetate, linalyl isobutyrate, α-acoreno, and geranyl isovalerate, with consequent higher antioxidant activity (FRAP, ABTS, and DPPH). However, these extracts showed lower yield and caryophyllene and humulene contents. Finally, PC3 separated E2-E5 (below the axis) from the other formulations (above the axis). This way, E2-E5, mainly E4 and E5, showed higher TBARS values.

The HCA of the data of yield, compounds, and antioxidant activity of the extracts (Fig. 3) showed a cophenetic correlation of 0.881, indicating the stability of the groups and reliability of the dendrogram. Based on the dendrogram, there were 4 groups: the first group with the E1, the second group con E2-E4, E7, and E8, the third group with E5, and the fourth group with E6 and E9-E11. These results demonstrate that E1 and E5 had different compositions and biological activities than the other evaluated extracts.

In general, PCA and HCA results confirm that mild conditions (lower temperature and pressure and low or high ethanol concentrations) may result in extracts with a higher concentration of compounds and more pronounced antioxidant potential in all antioxidant assays. Therefore, the recommendation is to use the conditions of E1 or E5. However, these extracts may show a lower yield, mainly E1.

3.5. Antimicrobial activity

The extracts showed inhibitory activity against Gram-positive and Gram-negative bacteria but were more effective against Gram-negative strains (Table 5). The results suggest that these extracts contain compounds with antibacterial potential. According to the obtained results, the extracts activity was as follows: E1 showed a MIC value of 5.0 mg/mL against *Proteus mirabilis*, E3 revealed a MIC of 10 mg/mL against *Escherichia coli* and *Proteus mirabilis*, E4 with a MIC of 10 mg/mL against *Enterococcus faecalis*, E6 with a MIC of 10 mg/mL against *Klebsiella pneumoniae*, E8 exhibiting a MIC of 5.0 mg/mL for *Proteus mirabilis*, and activity at 10 mg/mL against *Klebsiella pneumoniae* and *Listeria monocytogenes*, E9 with a MIC of 10 mg/mL for *Enterococcus faecalis*, E10 with MIC of 10 mg/mL for *Proteus mirabilis* and *Enterococcus faecalis*, and E11 with a MIC of 10 mg/mL for *Proteus mirabilis*. Thus, the hop residue extracts contain compounds with the antibacterial potential to inhibit

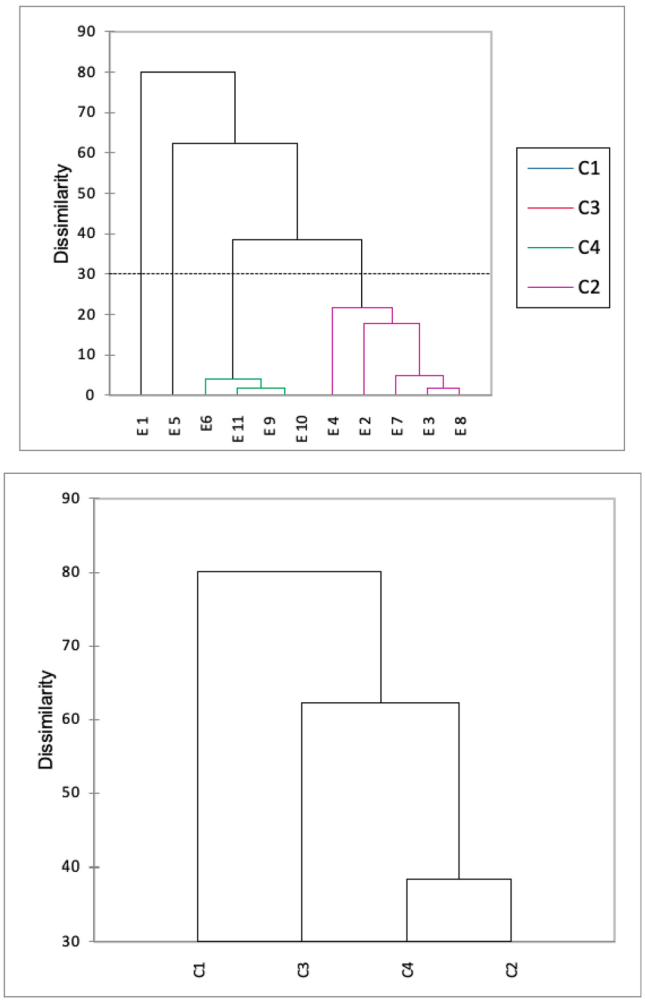

Fig. 3. HCA of the compounds, yield, and antioxidant activity.

the tested pathogens that cause intestinal, urinary, pulmonary, and meningeal infectious diseases. In addition, the Gram-negative bacterial strain *Proteus mirabilis* was the most sensitive to the hop residue extracts (E1, E3, E8, E10, E11).

Antimicrobial activity may be associated with lupulone and humulones in hop extracts. Such compounds have a hydrophobic characteristic, and the number and length of their side chains increase their lipophilic character, allowing an easier penetration on the bacterial cell wall. Their interaction with the inner wall damages cell structures may inhibit the active transport of sugars and amino acids and cause bacterial inhibition [6,40]. Differences in the type and concentrations of compounds found in the extracts can explain the different antimicrobial activity.

3.6. Cytotoxic activity

In order to confirm the safety of the extracts, primary pig liver cells (PLP2), which exhibit a marked cellular and physiological congruence with the respective human organ, were tested [41]. As can be seen in Table 6, the 11 extracts notably showed no hepatotoxicity for PLP_2 cells up to the maximum tested concentration of GI_{50} > 400 μg/mL. Alonso et al. [39] reported the same result for their extract of hop seed, stating that their extract does not cause liver cytotoxicity. Considering the above, it is suggested that the extracts from the brewery residue are potentially safe for products intended for human consumption. Nonetheless, further advanced tests are mandatory for their safe incorporation in pharmaceutical and nutraceutical products.

Table 5
Antibacterial potential of the hop cone residue extracts.

	Gram negative bacteria										Gram positive bacteria					
Extracts	*Escherichia coli*		*Klebsiella pneumoniae*		*Morganella morganii*		*Proteus mirabilis*		*Pseudomonas aeruginosa*		*Enterococcus faecalis*		*Listeria monocytogenes*		MRSA	
	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB
E1	> 10	> 10	> 10	> 10	> 10	> 10	5	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10
E2	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10
E3	10	> 10	> 10	> 10	> 10	> 10	10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10
E4	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	10	> 10	> 10	> 10	> 10	> 10
E5	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10
E6	> 10	> 10	10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10
E7	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10
E8	> 10	> 10	10	> 10	> 10	> 10	5	> 10	> 10	> 10	> 10	> 10	10	> 10	> 10	> 10
E9	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	10	> 10	> 10	> 10	> 10	> 10
E10	> 10	> 10	> 10	> 10	> 10	> 10	10	> 10	> 10	> 10	10	> 10	> 10	> 10	> 10	> 10
E11	> 10	> 10	> 10	> 10	> 10	> 10	10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10
Positive Controls																
	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB
Imipenem	< 0.0078	< 0.0078	< 0.0078	< 0.0078	< 0.0078	< 0.0078	< 0.0078	< 0.0078	0.5	1	n.t.	n.t.	< 0.0078	< 0.0078	n.t.	n.t.
Ampicillin	< 0.15	< 0.15	10	10	> 10	> 10	< 0.15	< 0.15	> 10	> 10	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15

MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentrations; n.t. – not tested; MRSA – Methicillin-resistant Staphylococcus aureus.

Table 6
Hepatotoxicity (GI_{50}, μg/mL) in non-tumor cells (PLP2).

Extract	PLP2 μg/mL	Positive control Ellipticine μg/mL
E1	> 400	1.4 ± 0.1
E2	> 400	1.4 ± 0.1
E3	> 400	1.4 ± 0.1
E4	> 400	1.4 ± 0.1
E5	> 400	1.4 ± 0.1
E6	> 400	1.4 ± 0.1
E7	> 400	1.4 ± 0.1
E8	> 400	1.4 ± 0.1
E9	> 400	1.4 ± 0.1
E10	> 400	1.4 ± 0.1
E11	> 400	1.4 ± 0.1

4. Conclusion

As a novel finding, this study presents the effectiveness and high yield of modifier-enhanced supercritical CO2 extraction in retrieving compounds from hop cone residue used in craft beer production. Furthermore, employing the suggested approach, the chemical composition and mass percentage of extracts procured from hop cone residues demonstrate a similar quantity and quality to virgin hop cones, categorizing them as a substantial co-product. The extracted compounds showed potential antioxidant and antimicrobial activity and proved safe according to hepatotoxicity screening assays. Thus, using this by-product in the pharmaceutical, nutritional, and cosmetic sectors is feasible and contributes to reducing the environmental impact and creating a more sustainable production chain. The study found that lower temperatures (40 °C) and pressures (10 MPa), as well as low or high ethanol percentages (5–10%), resulted in higher concentrations of certain compounds, including alpha-copaene, γ-elemene, nerolidyl acetate, linalyl isobutyrate, α-acorenol, and geranyl isovalerate, and increased antioxidant potential. The study also observed that the percentage of ethanol used as a co-solvent could influence the extract composition and biological activity.

CRediT authorship contribution statement

Conceptualization, Lucio Cardozo-Filho[e], José Eduardo Gonçalves· Methodology, Lucio Cardozo-Filho[e], José Eduardo Gonçalves[a,b], Lillian Barros· Software, Tatiana Colombo Pimentel· Validation, Lucio Cardozo-Filho[e], Lucio Cardozo-Filho[e], José Eduardo Gonçalves[a,b], Carla Pereira[c,d]; Ricardo M. Calhelha[c,d]; Sandrina A. Heleno· Formal analysis, Lucio Cardozo-Filho[e], Tatiana Colombo Pimentel· Investigation, Rodrigo Sadao Inumaro[a]; Rhaira Fernanda Ayoub Casalvara[a]; Rúbia Carvalho Gomes Corrêa[a,b,c], Carla Pereira[c,d]; Ricardo M. Calhelha[c,d]; Sandrina A. Heleno[c,d.] Resources, Lucio Cardozo-Filho[e], José Eduardo Gonçalves[a,b], Lillian Barros· Data curation, Rodrigo Sadao Inumaro[a]; Rhaira Fernanda Ayoub Casalvara[a]; Rúbia Carvalho Gomes Corrêa[a,b,c], Carla Pereira[c,d]; Ricardo M. Calhelha[c,d]; Sandrina A. Heleno· Writing – original draft, Lucio Cardozo-Filho[e], Eduardo Gonçalves[a,b],Tatiana Colombo Pimentel· Writing –breview & editing, Lucio Cardozo-Filho[e], José Eduardo Gonçalves[a,b], Lillian Barros[c,d],Tatiana Colombo Pimentel· Visualization, Lucio Cardozo-Filho[e], José Eduardo Gonçalves[a,b], Lillian Barros[c,d],Tatiana Colombo Pimentel. Supervision, Lucio Cardozo-Filho[e], José Eduardo Gonçalves· Project administration, Lucio Cardozo-Filho[e], José Eduardo Gonçalves[a,b], Lillian Barros. Funding acquisition, no available.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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