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Supercritical CO_2 extraction of bioactives from *P. halepensis* petals: Process modeling, mass transfer, and bioactivity characterization

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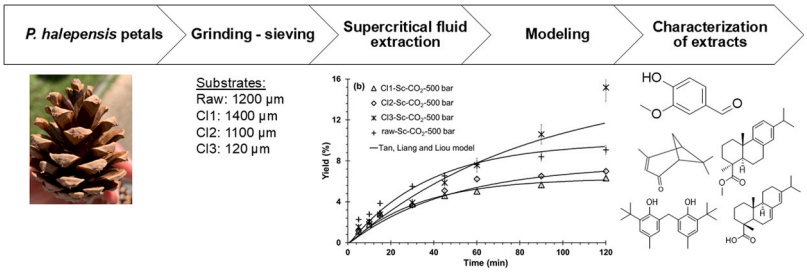
HIGHLIGHTS

- Pinecone extracts were obtained by supercritical fluid extraction (SFE) with CO_2.
- Tan, Liang and Liou model successfully fitted the extraction kinetics.
- Extracts by CO_2 with water exhibited significant antioxidant activity (DPPH test).

GRAPHICAL ABSTRACT



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ABSTRACT

The extraction of bioactive compounds from *P. halepensis* petals was carried out using supercritical carbon dioxide ($Sc\text{-}CO_2$) under varying operational conditions, including extraction time, pressure (300–500 bar), co-solvent type (water or ethanol), and particle size (120–1400 µm), and compared to conventional techniques such as Soxhlet extraction and maceration. The results showed that $Sc\text{-}CO_2$ extraction at 300–500 bar achieves ~80 % recovery of bioactive compounds within the first 30 min. Tan, Liang and Liou model successfully fitted the extraction kinetics. Furthermore, all extracts demonstrated moderate to high anticancer activity against LS174t and HCT116 cell lines compared to tamoxifen (a well-known anticancer standard). Extracts obtained by $Sc\text{-}CO_2$ with water as a co-solvent exhibited significant antioxidant activity against DPPH free radical; however, their antioxidant activities were notably lower than those obtained through conventional extraction methods. Finally, 38 molecules were identified by HPLC-DAD and 24 by GC-MS. The originality and novelty of this study are a) First-time application of $Sc\text{-}CO_2$ to extract bioactive compounds from *P. halepensis* petals; b) Identification of new chemical compounds (seven detected for the first time in this species) as a novel contribution.

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Symbols

a	specific surface of the solid phasem^{-1}
AARD	average absolute relative deviation%
Bi	Biot number/
c_f*	equilibrium concentration of extract in the fluid phaseg/L
c_F	concentration of extract in the fluidg/L
Cl1	coarse particle class/
Cl2	intermediate particle class/
Cl3	finer particle class/
$\bar{c}_s$	mean concentration of extract components in the solid phaseg/L
d	mean diameterm
D	diffusion coefficient in the solid phasem^2/s
D_{ax}	axial dispersion coefficientm^2/s
D[3,4]	volume-weighted mean diameterm
k_{oG}	overall mass transfer coefficient for the gas phasem/s
K	equilibrium distribution coefficient between liquid and solid phase/
PA-P	*P. halepensis* petal/
raw	raw material before sieving/
Re	Reynolds number/
Sc	Schmidt number/
Sh	Sherwood number/
ρ_s	density of the solid materialkg/m^3
t	time of extractionmin
u	void volume linear velocity of supercritical solventm/s
yield	extraction yield% w/w
z	coordinate in axial directionm
β_F	mass transfer coefficient for the fluid phasem/s
ε	porosity of the fixed bed/

1. Introduction

P. halepensis, native to the Mediterranean region, is prevalent in semi-arid and arid zones, covering an area of approximately 3.5 million hectares [1]. Historically, different parts of this species have been extensively used in traditional medicine across various cultures. The seeds of *P. halepensis* are traditionally used to prepare sweet pudding. Moreover, its essential oil has been employed for its antiseptic, antitubercular, and antirheumatic properties [2]. The resin and extracts of *P. halepensis* have been applied in the treatment of wounds, respiratory disorders, and skin infections. Additionally, the compounds identified in *P. halepensis* have been found to possess a range of therapeutic properties, including anti-inflammatory, anticoagulant, antimicrobial, antibacterial, antifungal, antioxidant, and anticancer activities [3,4].

Supercritical fluid extraction (SFE) is an advanced technique for recovering and isolating plant extracts, offering superior quality compared to traditional methods [5]. SFE yields solvent-free, high-value extracts under mild conditions, making it ideal for natural product extraction [6]. Supercritical carbon dioxide (Sc-CO_2) is the most commonly used SFE solvent due to its non-toxicity, non-flammability, low cost, easy removal, and high extraction efficiency. Its low polarity limits polar compound extraction, which can be improved by adding a polar co-solvent [7].

Pinecones, after maturing and falling from trees, contribute to forest waste and generate an unvalorized agri-resource. In France, approximately one million tonnes of pinecones are produced annually, contributing to the growing forest bio-waste (https://franceboisforet.fr accessed 21 December 2024). Pinecones are a valuable source of commercially important products, including pine essential oil and chemicals [8]. Additionally, polysaccharides extracted from pinecones have shown potential for various applications [9]. Recently, pinecones and their extracts have been explored for industrial wastewater treatment as natural coagulants [10]. While extraction methods such as hydrodistillation, maceration, Soxhlet, and supercritical CO_2 have been applied to pinecones [11], there is limited research on the extraction of essential oils and phytochemicals from specific pinecone species, particularly *P. halepensis*. The recovery of valuable compounds from *P. halepensis* pinecones holds significant promise, as they are known to be rich in bioactive substances such as proteins, polysaccharides, and polyphenols, which have proven benefits for food, feed, cosmetic, and health product applications. Our previous research [12] indicates that the petal fraction of pinecones, particularly from *P. halepensis*, exhibits superior antioxidant, anticancer, and antidiabetic activities compared to the core fraction or whole pinecone, highlighting the potential of petals for further exploration and utilization. Moreover, Chammam et al. [12] reported that aqueous extracts from *P. halepensis* petals exhibited significant anticancer activity, inhibiting 57 % of HepG2 (hepatic carcinoma) cells and 66 % of HeLa (cervical carcinoma) cells, while showing low toxicity (<15 % inhibition) toward non-cancerous HEK-293 (human embryonic kidney) cells, indicating that the petal extracts are safe and well tolerated. Furthermore, pinecones have traditionally been used in Japanese folk medicine, particularly for treating gastric cancer, as well as for moisturizing the lungs, relieving cough, and reducing fever, reflecting their non-toxic nature [12].

To the best of our knowledge, no study has yet reported on the extraction of bioactive compounds from *P. halepensis* petals using supercritical fluid extraction (SFE) techniques. In our previous research, we demonstrated the potential of *P. halepensis* petals (PA-P) as a promising matrix for their remarkable biological properties and phytochemical richness, surpassing other pine species and fractions when extracted using conventional methods such as maceration [12,13]. The aim of this study is to explore green and advanced extraction techniques by utilizing SFE to obtain high-quality extracts from PA-P and to evaluate the influence of key processing parameters, such as pressure, granulometry, and co-solvent addition on the global yield, composition, bioactivities, and extraction kinetics. Furthermore, extraction kinetics were modeled to provide a deeper understanding of the process. The results of SFE were compared with conventional techniques, including Soxhlet extraction with organic solvents (hexane and ethanol) and maceration in water.

2. Materials and methods

2.1. Materials

The pinecones of *P. halepensis* were collected at the optimal stage of maturity, in December 2016 from Bizerte (latitude: 37°16′27″, longitude: 9°52′26″, northern Tunisia), which is one of the ecologically optimum zones for pinecone production in Tunisia. According to our previous study, PA-P presents chemical and biological properties more than core fractions and full pinecones [12], which motivates us to focus on this study only on the petal fractions. The procedures for separating, drying, milling, and sieving of the PA-P and the characterization of the raw material have been described in our previous publication [13]. PA-P exhibited a moisture content of 9.70 % on a dry basis and 8.84 % on a wet basis. The density of the substrate PA-P was determined using the gravimetry-volume method to be 1120 ghm/L [13], and the particle bed porosity was estimated at 0.20. The ground PA-P was sieved using a vibrating sifter (D411, Controls Milano, Italy; 220 V, 50 Hz, 370 W; SN: 92092405). Four square-mesh sieves (NF X11–501, stainless steel frame, Saulas, Paris, France) (3, 2, 1, and 0.12 mm) were employed to separate the particles based on size. The sieving procedure produced five distinct

particle size fractions (F1 to F5), with corresponding mass percentages of 0.02, 0.02, 35.73, 54.39, and 9.84 % w/w, respectively. Fractions F1 and F2, due to their negligible mass, were grouped with F3 to form Class 1 (Cl1, d > 1 mm). Fraction F4 was assigned to Class 2 (Cl2, 0.12 < d < 1 mm), and F5 to Class 3 (Cl3, d < 0.12 mm). The volume-weighted mean diameter, D[3,4], was 1200, 1400, 1100, 120 μm for the raw (the unsieved matter; Cl1 +Cl2 +Cl3), Cl1, Cl2, and Cl3, respectively (Table 1).

Carbon dioxide (CO_2; 99.95 % purity) was provided by Nippon Gases (Hamburg, Germany). Hexane (≥95 %) and ethanol (99 %) were purchased from Roth (Karlsruhe, Germany). The analytical standards used to identify the non-volatile compounds present in the extracts were purchased from Sigma Aldrich (Saint-Quentin, France): trihydroxyethylrutin; rutin; catechin; 3,4-dihydroxy-5-methoxybenzoic acid; quercetin, 3-β-D-glucoside; polydatin; 2,4-dihydroxycinnamic acid; ellagic acid; (±) synephrine; chlorogenic acid; gallic acid; (−)-epicatechin; gallocyanine; brilliant yellow; methyl 3,5-dihydroxybenzoate; 3-amino-4-hydroxybenzoic acid; 3,4-dihydroxycinnamic acid (caffeic acid); sinapic acid; trans-3-hydroxycinnamic acid; p-coumaric acid; trans-ferulic acid; 4,7-dihydroxycoumarin; 7-hydroxycoumarin-3-carboxylic acid; 7-hydroxycoumarin-3-carboxylic acid; methyl 4-hydroxybenzoate; myricetin; 6-hydroxycoumarin; coumarin; 7-hydroxy-4-methyl-3-coumarinylacetic acid; 3-cyanoumbelliferone; isopropyl 3,4,5-trihydroxybenzoate; resveratrol; 4-hydroxy-3-methoxycinnamic acid; 2-hydroxycinnamic acid; quercetin; ethyl 3,4-dihydroxycinnamate; 7,8-dihydroxy-2,2-dimethylchromane-6-carboxylic acid; trans-cinnamic acid; 3,4-dihydroxybenzoic acid methyl ester; 4-ethyl-7-hydroxy-8-methyl-2H-chromen-2-one; α-cyano-4-hydroxycinnamic acid; naringenin; (±)-taxifolin; 7,3′-dihydroxyflavone; 2,4-dihydroxy-3,6-dimethylbenzoic ac-id; 3-cyano-7-hydroxy-4-methylcoumarin; (±)-6-hydroxy-2,5,7,8-tetramethylchroumane-2-carboxylic acid; butyl gallate; 6-hydroxyflavone; baicalein; ethyl 3,5-dihydroxybenzoate; ethyl trans-2-hydroxycinnamate; kaempferol; 5,8-dihydroxy-1,4-naphthoquinone; ethyl 4-hydroxy-3-cinamate; 7-hydroxy-4-phenylcoumarin; 2-chloro-3-(4-hydroxy-phenylamino)-(1,4) naphthoquinone; 5-hydroxy-4′-methoxylflavone; chrysin; warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin); icariin; 3′-hydroxy-a-naphthoflavone; 3-tert-butyl-4-hydroxybenzoic acid; 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone; 7-hydroxyflavone; β-carotene; lutein; 4-hydroxytamoxifen; 5,7-dihydroxy-4-propyl-coumarin; shikonin; 3′-hydroxy-6-methylflavone; 7-hydroxy-4-(trifluoromethyl) coumarin; 5-hydroxyflavone; isobutyl 4-hydroxybenzoate; 3,3′,4′-trimethoxyflavone; butyl 4-hydroxybenzoate; 7-hydroxy-3′,4′,5′-trimethoxy-alpha-naphthoflavone; 3′-hydroxy-b-naphtoflavone; 3,3′-dimethoxyflavone; 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone; 3,6,3′-trimethoxyflavone; 3,7-dimethoxyflavone; 5-hydroxy-3′-metho-xyflavone; xan-thurenic acid; 4′,5′-dimethoxy-2′-hydroxy-4-methylchalcone; rosmarinic acid; (z)-3-(3-ethoxy-4-hydroxyphenyl)-2-phenyl-acrylic acid; 2-chloro-3-(3,5-di-tert-bu-tyl-4-hydroxyphenyl)(1−4)naphtoquinone; hamamelitannin; 3,4-dihydroxy-5-methoxycinnamic acid; and 5-hydroxy-7-((3-methylbenzyl)oxy)-2-phenyl-4h-chromen-4-one.

2.2. Extraction processes

2.2.1. Soxhlet extraction

The extraction of compounds was performed using a Soxhlet apparatus (Complete Soxhlet Extractor with 4-position heating system, 250 mL, Behrotest R254S with stopcock, Behr Labor-Technik, Düsseldorf, Germany) employing 95 % pure ethanol and hexane as solvents. Approximately 4 g of the PA-P sample, with a particle size of 1400 μm (Cl1), was placed in a cellulose Soxhlet cartridge. The 250 mL distillation flask was filled with 100 mL of solvent (hexane and ethanol) and assembled with the Soxhlet extractor and reflux condenser. The extraction was performed for 6 h with hexane and 8 h with ethanol, continuing until the solvent showed no further color change from extracted compounds, indicating completion. The extraction was carried out at atmospheric pressure and temperatures of 69 and 78°C, corresponding to the boiling points of hexane and ethanol, respectively. After extraction, the solvent was evaporated under reduced pressure using a vacuum oven (Thermo Scientific, SN: 42069766, 50/60 Hz, 230 V, 7.4 A, 1.7 kW, Thermo Fisher Scientific, Waltham, MA, USA) at 5 mbar and 35°C for 6 h. The resulting dry extract was weighed and stored at −20°C for further analysis. The extractive yield was calculated as a percentage of the original dry-weight sample mass using Eq. (1):

$$\%yield = \frac{m_{de}}{m_{dm}} \quad x \quad 100 \tag{1}$$

Where: m_{de} represents the weight of dry extract (g) and m_{dm} denotes the weight of dry substrate material (g).

Conventional techniques, such as Soxhlet extraction and maceration, were used to compare the yields and quality of extracts with those obtained by Sc-CO_2 and modified Sc-CO_2. Solvents for Soxhlet extraction were selected based on polarity: hexane (non-polar) was used for lipophilic compounds, compared to Sc-CO_2, which shares similar non-polar characteristics, while ethanol (polar) targeted hydrophilic compounds and was compared to Sc-CO_2 + ethanol, which has similar polarity. Due to experimental limitations, we opted for maceration in water instead of Soxhlet extraction with water.

Table 1
Semi-batch extraction conditions from P. halepensis petals (PA-P).

PA-P samples	Techniques	Solvents	Conditions						
			P (bar)	T (°C)	Q (g/min)	x (% w:w)	t (min)	D[3,4]	m (g)
Cl1	SFE	CO_2	300, 400, 500	35	4	N/A	120	1413 ± 286	4 ± 0.8
		CO_2+EtOH	200	35	4	5, 10	30, 60, 90		
		CO_2+H_2O	200	35	4	0.2	30, 60, 90		
	Soxhlet	Hexane	P_{atm}	69	N/A	N/A	480		
		EtOH	P_{atm}	78	N/A	N/A	480		
	Maceration	H_2O	P_{atm}	20	N/A	N/A	1440		5 ± 0.8
Cl2	SFE	CO_2	500	35	4	N/A	120	1097 ± 357	4 ± 0.8
		CO_2+EtOH	200	35	4	10	90		
		CO_2+ H_2O	200	35	4	0.2	90		5 ± 0.8
Cl3	SFE	CO_2	500	35	4	N/A	120	121 ± 31	4 ± 0.8
		CO_2+EtOH	200	35	4	10	90		
		CO_2+H_2O	200	35	4	0.2	90		5 ± 0.8
raw	SFE	CO_2	500	35	4	N/A	120	1208 ± 481	4 ± 0.8
		CO_2+EtOH	200	35	4	10	90		
		CO_2+H_2O	200	35	4	0.2	90		5 ± 0.8

P (bar): Pressure; T (°C): Temperature; Q (g/min): Flow rate; x (% w:w): Weight % of co-solvent; t (min): time; d (mm): Particle size; m (g): sample mass; SFE: Supercritical Fluid Extraction; EtOH: ethanol; H_2O: distilled water; PA-P: *P. halepensis* petal; Cl1: Class 1, Cl2: Class 2; Cl3: Class 3; raw: unsieved matter (Cl1 +Cl2+Cl3); D[3,4]: volume-weighted mean size particle; N/A:

2.2.2. Maceration extraction

A 5 g portion of each sample was subjected to maceration in 50 mL of distilled water for 24 h at 20 °C, with continuous stirring at 150 rpm as described in our previous publication [12]. Maceration in water was used as a reference method to compare the yields and quality of extracts with those obtained by Sc-CO_2 + water. The resulting mixtures were subsequently filtered through Whatman filter paper (20 μm) and evaporated using a rotary evaporator (IKA, RV 10 auto V, Staufen, Germany) under vacuum at a reduced pressure and a temperature of 35 °C. The extraction yield was determined using Eq. (1).

2.2.3. Supercritical fluid extraction

The impact of granulometry (raw and sieved classes) and pressure levels (300, 400, and 500 bar) on the extraction kinetics was evaluated using Sc-CO_2 at a constant flow rate of 4 g/min and a fixed temperature of 35°C over 120 min. The CO_2 flow rate was fixed at 4 g/min due to technical limitations of the extraction apparatus. Additionally, the extraction temperature was maintained at 35°C to preserve the stability of thermolabile (heat-sensitive) compounds in the PA-P matrix, preventing thermal degradation during the process. Furthermore, the impact of granulometry (raw PA-P and sieved classes) and extraction time (30, 60 and 90 min) were studied for modified Sc-CO_2 extraction by adding polar co-solvents, such as ethanol (EtOH) and distilled water to enhance the solubility and recovery of polar bioactive compounds, particularly polyphenols, at 35 °C and 200 bar. Adding a small percentage of co-solvent completely alters the behavior of the supercritical fluid and enhances the yield even under moderate operating conditions. Therefore, a mild pressure of 200 bar can be taken advantage of to reduce operational costs.

As shown in Fig. 1, the unit consisted of two extractors (25 cm capacity; 1.4 cm internal diameter (i.d.) and 16 cm effective height) and (50 cm capacity; 1.4 cm internal diameter (i.d.) and 30 cm effective height) and one separator. The whole system was heated to the experimental temperature before starting the experiments (1). The CO_2 is pumped to reach a pressure exceeding critical pressure (3). It then flows through a heat exchanger, raising its temperature above critical temperature (4). Under supercritical conditions, the CO_2 interacts with crushed PA-P within the extractor (+). The Sc-CO_2 laden with extract undergoes a pressure drop to ambient pressure and a temperature-dependent on operating conditions. Expansion valve is heated in the range of 90–130 °C, to prevent the freezing of CO_2 caused by sudden expansion during separation in the separator (6). The samples were collected in glass flasks (7) (previously weighed). The vial was changed at specific operation times to determine the impact of the time on the extraction yield. About 4 g of each sample was placed in the extractor. For extractions using ethanol (EtOH) as a co-solvent, 5 and 10 % (w/w) relative to CO_2 were used at 200 bar, with their flow rate adjusted by an HPLC pump (2) (Fig. 1). In the case of water as a co-solvent, due to its low solubility in Sc-CO_2 (0.2 % at 200 bar and 35 °C [14]), the water reservoir was initially situated in void space below the raw material (Fig. 2). This physical separation ensures that the sample is never in direct contact with liquid water, which induces a biphasic state of the saturated liquid and the saturated gas (above the liquid phase). In this setup, Sc-CO_2 first becomes saturated with water at the bottom of the vessel, then Sc-CO_2+ 0.2 % water traverses the perforated plate and contact the substrate, enabling the extraction of polar compounds. The

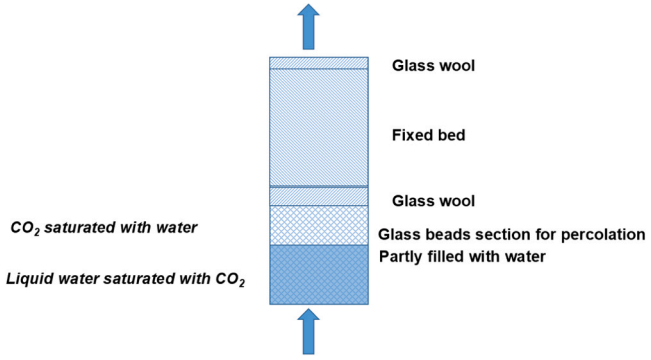


Fig. 2. Experimental setup for water-modified CO_2 extraction.

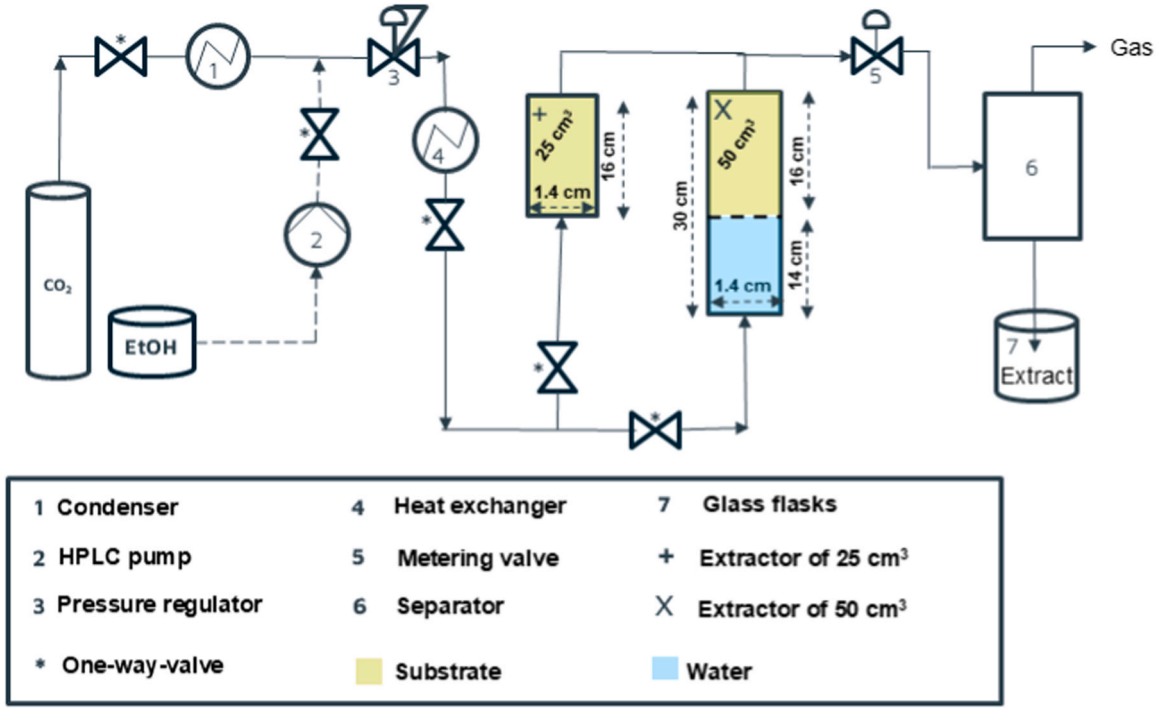


Fig. 1. Flowsheet of the supercritical carbon dioxide extraction unit; CO_2: Carbon Dioxide; EtOH: Ethanol; (1) condenser; (2) HPLC pump; (3) pressure regulator; (4) heat exchanger; (5) metering valve; (6) separator; (7) Glass flask; (*) one-way-valve; (+) extractor of 25 cm; (X) extractor of 50 cm; ( ) substrate; ( ) water.

method is similar to the percolation method for measuring single compounds phase equilibria (operational solubilities) in Sc-CO_2, provided a sufficient low flow rate is attributed to the CO_2-stream.

The mass of the extracts was determined gravimetrically after solvent evaporation in a vacuum oven (Thermo Scientific, SN: 42069766, 50/60 Hz, 230 V, 7.4 A, 1.7 kW, Thermo Fisher Scientific, Waltham, MA, USA) at 5 mbar and 35 °C, until a constant weight was reached after 6 h. The extraction yield was determined using Eq. (1). The extraction conditions are reported in Table 1.

2.3. Modeling

The kinetics extraction results using Sc-CO_2 have been modeled by applying the model defined by Tan, Liang and Liou [15], presented by Brunner [16]. This model was previously used by Danielski et al. [17] and Phan Tai and Brunner [18] to fit oil extraction yields from rice bran and palm fruit, respectively. In this model, the solid particles are assumed to be simple spheres with a specific diameter. The simulation used the following differential equations (Eqs. 2–5), which were solved with the software "BatchSFE", developed by the Institute of Thermal Separation Processes of the Hamburg University of Technology:

Mass balance for the fluid phase:

$$\frac{\partial C_F}{\partial t} = D_{ax}.\frac{\partial^2 C_F(z)}{\partial z^2} - \frac{U_z}{\varepsilon}.\frac{\partial C_F(z)}{\partial z} - \frac{1-\varepsilon}{\varepsilon}.\frac{\partial \overline{c}(z)}{\partial t} \tag{2}$$

Mass transfer for the solid phase:

$$\frac{\partial \overline{C_s}(z)}{\partial t} = a.k_{oG}\left(C_F(z) - \overline{C_s}(z).\frac{K(\overline{C_s})}{\rho_s}\right) \tag{3}$$

Adsorption equilibrium of substrate between fluid and solid phase:

$$K(\overline{C_S}) = \frac{C_F^*}{\overline{C_s}} \tag{4}$$

Mass transfer parameters at surface:

$$\frac{\beta_F}{k_{0G}} = 1 + \frac{Bi.K(\overline{c})}{6} \; with \; Bi = \frac{\beta_F.R}{D} \tag{5}$$

The diffusion, desorption, and dispersion parameters were estimated by performing least squares fit (AARD %) on the experimental data.

The influence of hydrodynamics, defined by the Reynolds number (Re), on mass transfer, characterized by the product of the Sherwood number (Sh) and the Schmidt number (Sc), was determined using various semi-empirical models of the form of Eq. (6)

$$\frac{Sh}{\sqrt[3]{Sc}} = A\mathrm{Re}^n \quad + B \quad , \text{with} Sh = \frac{\beta_F.d}{\mathrm{D}} \quad \text{and} Sc = \frac{\mu}{\rho.D} \tag{6}$$

Where: A, B and n were constants of the models.

2.4. Chemical and biological analyses

2.4.1. Analyses of reducing sugar content (RSC)

The quantification of reducing sugars (RSC) in extracts obtained through SFE, Soxhlet, and maceration extractions was carried out using the 3,5-dinitrosalicylic acid (DNSA) method, as outlined in our previous study [12]. Briefly, 150 µL of each extract (350 mg/L) was combined with 150 µL of DNSA solution. Following this, 750 µL of deionized water was added after the mixture was incubated at 100°C for 5 min under constant stirring. Absorbance was measured at 530 nm. A reference blank (sodium potassium tartrate in 2 M NaOH instead of DNSA) and negative control (where the extracts were replaced with dimethyl sulfoxide, DMSO) were included. The limit of detection (LOD), defined as the lowest analyte concentration that produces a signal distinguishable from the baseline noise with a signal-to-noise ratio (S/N) of 3, was calculated to be 0.01 mg/g. The limit of quantification (LOQ), corresponding to the lowest concentration that can be quantitatively determined with suitable precision and accuracy (S/N = 10), was determined to be 0.03 mg/g. This indicates that concentrations of reducing sugar above the LOD and LOQ values are sufficiently high for accurate detection and quantification. The sugar content was reported as milligrams of glucose equivalent per gram of dry weight (mg GE/g DW).

2.4.2. Analyses of total phenolic content (TPC)

The total phenolic content (TPC) in extracts obtained through SFE, Soxhlet, and maceration extractions was carried out using the Folin–Ciocalteu (F-C) colorimetric method described in our previous work [12]. In an alkaline condition, created by sodium carbonate (Na_2CO_3), the phenolic compounds in the samples were oxidized, resulting in the reduction of the Folin–Ciocalteu reagent's phosphotungstic and phosphomolybdic acids, forming a blue-colored complex. The intensity of the blue color, which correlates with the phenolic content, was measured with a microplate reader (Multiskan Go, F1–01620, Thermo Fisher Scientific, Vantaa, Finland) at 765 nm. Under the experimental conditions used, the LOD was calculated to be 0.001 mg/g, while the LOQ was determined to be 0.002 mg/g. This means that total polyphenol concentrations above LOD and LOQ can be accurately detected and quantified.

The TPC was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW), based on the regression equation derived from the standard calibration curve of gallic acid concentrations ranging from 0 to 115 mg/L.

2.4.3. Determination of antioxidant activity (AOA)

The antioxidant activity of the extracts obtained through SFE, Soxhlet, and maceration extractions was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay described in our previous study [12]. Ascorbic acid was utilized as reference (4 µg/mL). A volume of 20 µL from each extract, prepared at a concentration of 0.5 mg/mL, was mixed with 180 µL of a methanolic DPPH solution (0.2 N) in a 96-well microplate (Micro Well; Thermo Fisher Scientific, Illkirch, France). The mixture was incubated at 25 °C for 25 min. Absorbance was subsequently recorded at 524 nm using a microplate reader (Multiskan Go, F1–01620, Thermo Fisher Scientific, Vantaa, Finland). The percentage of DPPH inhibition was calculated using Eq. (7).

$$\%inhibition = \quad 100 \times \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \tag{7}$$

Where A_{blanc} is the absorbance of the solvent and DPPH radical when no samples are present and A_{sample} is the absorbance of the sample and DPPH radical.

2.4.4. Determination of anticancer activity (ACA)

The anticancer activity of the extracts obtained through SFE, Soxhlet, and maceration was evaluated by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test as detailed in our previous publication [12]. Two cancer cell lines were tested: a human colon adenocarcinoma type B (LS174t) from a female and a human colon adenocarcinoma (HCT116) from a male. Human embryonic kidney cells (HEK-293) were used to assess the toxicity effect of extracts. The LS174t cell lines were cultured in RPMI 1640 medium (Roswell Park Memorial Institute), HCT116 cell lines were grown in advanced DMEM (Thermo Fisher Scientific), and HEK-293 cell lines were cultured in high-glucose DMEM (Dulbecco's Modified Eagle's Medium, France). Each culture medium was supplemented with decomplemented fetal bovine serum (10 %), non-essential amino acids (1 %), and antibiotics, including penicillin, streptomycin, and gentamicin. As a reference, Tamoxifen was used at 1, 10, and 100 µM. Cells were treated in triplicate with each extract (50 µg/mL) and incubated for 48 h at 37 °C. Absorbance at 605 nm was recorded using a microplate reader (Mullikan Go, F1–01620, Thermo Fisher Scientific, Vantaa, Finland).

2.4.5. Identification of "non-volatile" compounds (HPLC)

The "non-volatile" compounds of the extracts obtained through SFE, Soxhlet, and maceration were identified using HPLC-DAD (Thermo Scientific Accela pump, Accela PDA detector), as detailed in our previous publication [12]. An RP-C18 column (Phenomenex; Le Pecq, France, 25 cm × 4.6 mm, 5 µm) was used for the separation, and the flow rate was set at 0.5 mL/min. Acidified water (pH = 2.65) was used as solvent A in the mobile phase, and a solution of acidified water and acetonitrile (H_2O/ACN, 20/80 v/v) was used as solvent B.

The extracts were dissolved in acidified water/ACN (20:80 v/v) at 10 mg/mL. Compounds were identified at 280 nm by comparing their retention times and maximum absorbance (lambda max) data with reference standards mentioned in Section 2.1.

2.4.6. Identification of "volatile" compounds (GC-MS)

The "volatile" compounds of the extracts obtained through SFE, Soxhlet, and maceration were identified using GC-MS as detailed in our previous publication [12]. Extracts were dissolved at 3 mg/mL in ACN. The analysis was performed on a Saturn 2000 gas chromatograph (Les Ulis, France) with a DB-5MS fused silica capillary column (5 % phenylmethylpolysiloxane, 30 × 0.25 mm, 0.25 µm film thickness). Hydrogen served as the carrier gas. 5 µL per extract were injected to analyze.

The identification of compounds in the extracts was carried out by comparing their mass spectra to those in the NIST08 database (National Institute of Standards and Technology, https://www.nist.gov/, MS library version 2.4, build 25 March 2020).

2.4.7. Statistical analysis

All experiments were conducted in triplicate. The data were expressed as mean values ± standard deviations. The confidence limits were set at $p < 0.05$ and calculated according to the ANOVA test using the Statistical Package for the Social Sciences (SPSS) 22 (version IBM. 22.0. 2013; San Francisco, CA, USA). The difference between the extraction techniques, solvents, and granulometry was determined using Tukey's test.

3. Results and discussion

3.1. Extraction kinetics using pure Sc-CO_2 and modeling

Fig. 3 illustrates the impact of pressure (ranging from 300 to 500 bars) and granulometry on the extraction kinetics of bioactive compounds from PA-P using Sc-CO_2 at 35 °C and 4 g/min. The extraction yield showed a rapid increase during the first 20 min, followed by a gradual decrease, reaching a plateau after 60 min.

As illustrated in Fig. 3a, within the pressure range of 300–500 bar, the extraction yields obtained using Sc-CO_2 were generally independent of pressure during the initial 20 min. However, beyond this period, a significant influence of pressure on the overall extraction yield was observed, increasing from 4.52 % to 6.32 % from 300–500 bar after 120 min. Furthermore, Sc-CO_2 extraction at 300–500 bar achieves ~40 % recovery of bioactive compounds within the first 20 min and nearly 80 % by 30 min, based on the maximum yield obtained at the plateau after 60 min of extraction. Comparing the overall yields obtained by Sc-CO_2 extraction and Soxhlet extraction with hexane, the yields achieved by Sc-CO_2 extraction after 20 min at 35°C and pressures between 300 and 500 bar were higher (ranging from 3 % to 6.32 %) than those obtained by Soxhlet extraction with hexane (2 %) after 480 min at 69°C and atmospheric pressure. The increase in yield with pressure could be attributed to the higher Sc-CO_2 density, which enhanced its solvent power for various compounds (lipids, polyphenols, etc.). As pressure increased, heavier or less volatile compounds became more accessible to Sc-CO_2.

In comparison with the literature, previous scientific research on Sc-CO_2 extraction from *P. halepensis* is limited, particularly regarding the

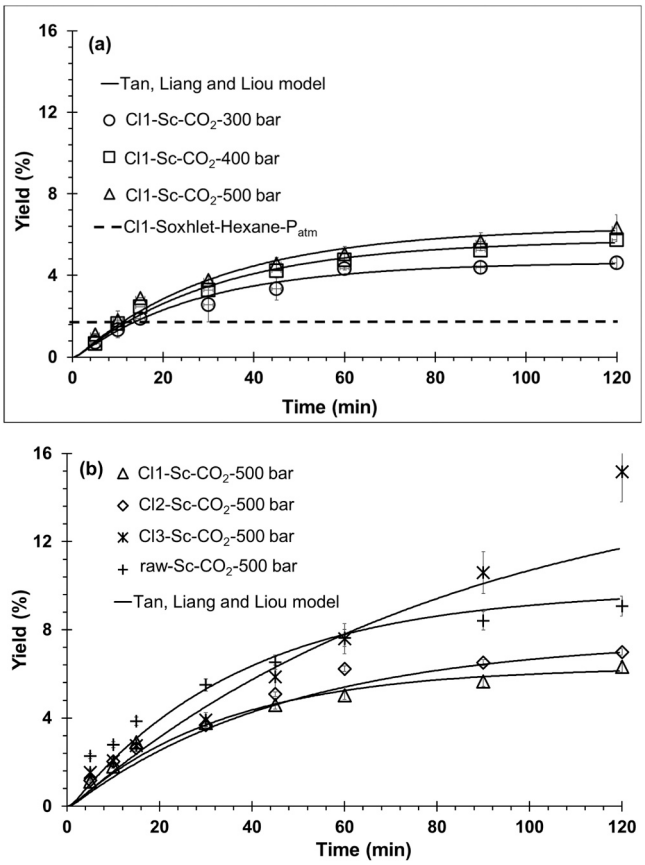


Fig. 3. Evaluation of the impact of (a) pressure and (b) granulometry on the kinetic extraction yields from P. halepensis at constant temperature (35°C) and flow rate (4 g/min) using pure Sc-CO_2. Extraction. (◯) Cl1 (1400 µm) at 300 bar; (▭) Cl1 (1400 µm) at 400 bar; (△) Cl1 (1400 µm) at 500 bar; (◇) Cl2 (1100 µm) at 500 bar; (+) raw (1200 µm) at 500 bar; (x) Cl3 (120 µm) at 500 bar. The results are expressed as means ± SD (n = 3).

petal fraction. The study by Nuralın et al. [19] concluded that the oil yield obtained by Sc-CO_2 from *P. brutia* cones significantly increased as the operating pressure was raised from 140 to 220 bar. The highest yield (4.18 %) was achieved at 220 bar, 35°C, after 120 min, with an average particle diameter of 1 mm. These results are consistent with ours. Similar trend of the impact of pressure was observed by Tamkutė et al. [20] in the range from 250 to 550 bar to extract bioactive compounds from cranberry pomace by SFE. Furthermore, the study by Kim et al. [21] demonstrated that the yields of bioactive compounds extracted from *Cannabis sativa L.* using modified Sc-CO_2 were higher than those obtained by pure Sc-CO_2 extraction and conventional methods such as maceration and Soxhlet, which is consistent with our results.

Given that the highest yield by Sc-CO_2 was obtained at 500 bar, the effect of granulometry on the extraction yield of PA-P (raw, Cl1, Cl2, and Cl3 with D [3,4]: 1200, 1400, 1100, and 120 µm, respectively) was studied at this pressure (500 bar), 35 °C and 4 g/min for 120 min (Fig. 3b). In addition to the solubility behaviour of solutes, the extraction yield depends on Sc-CO_2 diffusion within the plant matrix and solute release, making the plant material's physical structure a critical factor. Extraction kinetics indicate that after 20 min, granulometry has a significant impact on extraction yield ($p < 0.05$). Smaller particles result in higher yields, with the highest yield (16 %) achieved after 120 min using Cl3, which has the finest particles (120 µm).

Similar trend of particle size influence was reported by Pavlić et al. [22] in the range from 400 to 800–200–400 µm to extract bioactive compounds from raspberry seed using SFE. Numerous researchers have explored the influence of particle size on extraction yield using SFE and

have observed that the amount of extractable material increases as the particle size is reduced, attributing this observation to the associated increase in specific surface area [17,23,24]. Milling to reduce particle size could increase specific surface area and disrupt cell walls of the material, potentially leading to higher yields of bioactive compounds from finer particles than coarser particles, due to limited diffusion through intact cell walls [25].

Furthermore, as illustrated in Fig. 3, the results demonstrated that the model of Tan, Liang and Liou can be used to describe the extraction of bioactive compounds from PA-P. Only for Cl3, the deviations between experimental and calculated data are large. This can be explained by the approach of Pourmortazavi and Hajimirsadeghi [26], which suggests that too small particle sizes can compact the bed, leading to inhomogeneous extractions due to increased internal mass transfer resistance and fluid channeling effects in the fixed bed. This makes a modeling approach challenging.

As reported in Table 2, the Tan, Liang and Liou model was used to describe mass transfer phenomena during Sc-CO_2 extraction, with model parameters (diffusion, desorption, dispersion) estimated by least squares fitting as a function of pressure and granulometry. The model considers two mass transfer mechanisms: external transfer from the particle surface to the fluid (quantified by the fluid-phase mass transfer coefficient β_F) and internal diffusion from within the solid matrix (quantified by the diffusion coefficient D) (Fig. 4). Our results, consistent with Brunner [18], show that β_F is on the order of 10^{-6} m/s and D is on the order of $10^{-1\ 2}$ m^2/s, indicating that internal diffusion is the limiting step of the process. Extraction proceeds in two phases: a rapid initial phase dominated by surface desorption and external transfer, which is dictated by the solubility limit of the solute in Sc-CO_2, followed by a slower phase controlled by internal diffusion through micropores, similar to the falling-rate period observed in drying processes [27].

Regardless of pressure and particle size, axial dispersion (D_{ax}) can be considered constant, with low values on the order of 10^{-5} m^2/s. This indicates no significant effect of axial dispersion on the process, as described by Eq. (8) [28].

$$D_{ax} = 0.5 \quad .U_z. \quad d + D \tag{8}$$

The desorption of bioactive compounds could be enhanced by increasing the operating pressure, which can be attributed to the enhancement of solvent power. These observations were confirmed by Danielski et al. [17].

As reported in Table 2, reduction of particle size can lead to an increase in the diffusion coefficients (β_F and D) and the desorption constant (K) of bioactive compounds to be extracted. These findings suggest that a strong reduction of particle sizes not only increases the specific surface area but also affects the pore structure. This observation could be attributed to the influence of mechanical processing on the physicochemical structure of the matrix.

As illustrated in Fig. 5a, the impact of hydrodynamics, characterized by the Reynolds number (Re), on external mass transfer, described by

Table 2
Calculated parameters for Tan, Liang and Liou models.

PA-P samples	P (bar)	D_{ax} ($\times 10^{-5}$ m^2/s)	β_F ($\times 10^{-6}$ m/s)	D ($\times 10^{-12}$ m^2/s)	K	AARD %
Cl1 (1400 μm)	300	2.32	8.64	3.98	0.61	9.55
	400	2.32	3.50	3.71	1.43	9.95
	500	2.18	1.46	2.08	28.12	6.21
Cl2 (1100 μm)	500	2.18	3.71	2.34	32.33	4.96
Cl3 (120 μm)	500	2.18	4.95	2.37	39.98	15.59
Raw (1200 μm)	500	2.18	3.44	2.13	28.41	7.84

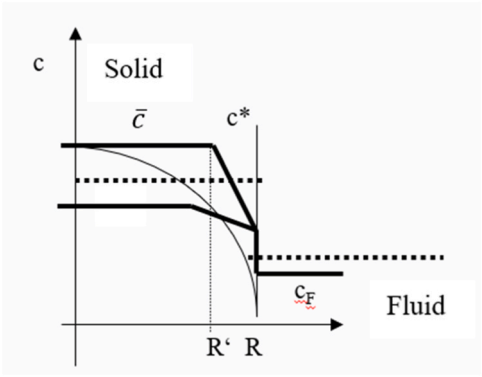


Fig. 4. Radial profile of solute concentration per unit time within the particle and at the phase transition interface.

the product of the Sherwood number (Sh) and the Schmidt number (Sc), fits acceptably well with the data and kinetic models for bulk oils proposed by del Valle and de la Fuente [29]. Although all the obtained values of the external mass transfer coefficient are close, a slight trend was observed with variations in pressure and granulometry (Fig. 5a). The highest value was observed with the coarser particle (Cl1) at the lowest operating pressure (300 bar) and the lowest was reported with the finer particle (Cl3) at the highest pressure (500 bar). Increasing pressure from 300 to 500 bar may decrease the velocity, which could reduce Reynolds and Sherwood numbers at a constant flow rate (4 g/min). Additionally, as particle size increases, diffusion decreases, which may lead to higher Sherwood and Reynolds numbers at the same flow rate, as described by Eq. (6).

As shown in Fig. 5.b, a good agreement is observed between our results and the literature, as well as the reference models applied to the extraction kinetics of oils by Sc-CO_2 in a fixed bed from various plant matrices, as proposed by del Valle and de la Fuente [29]. Furthermore, based on the positioning of our data relative to groups A and B in the adapted diagram, it can be inferred that the compounds solubilized by Sc-CO_2 from the PA-P class are predominantly volatile and liquid compounds under the extraction conditions applied (35 °C and 300–500 bar) [29]. These compounds were subsequently identified and characterized by HPLC (Section 3.3.5.) and GC–MS (Section 3.3.6.).

3.2. Impact of co-solvent on extraction yield

Carbon dioxide is a nonpolar molecule, whereas polyphenolic compounds are more polar [34]. Therefore, to effectively extract these target polar molecules, the addition of a polar co-solvent is recommended [35]. Polar co-solvents such as ethanol and water are commonly used in extraction since they are safe for humans and the environment, and used commonly in the pharmaceutical, cosmetic, and food industries [36].

As illustrated in Fig. 6, experiments on PA-P with Sc-CO_2 with co-solvents (ethanol and water in this study) were conducted at 200 bar, 35 °C, and a CO_2 flow rate of 4 g/min, with different times (30, 60, and 90 min), weight % of EtOH (5 and 10 %), and granulometry.

By comparing the co-solvent effects, it is possible to deduce that at a constant temperature, the co-solvent impact increases with increasing weight %, extraction time, and decreasing granulometry. The highest yield was reported to Cl3 for Sc-CO_2+ 10 % EtOH and Sc-CO_2+ 0.2 % H_2O with 12 and 14 % (w/w) respectively after 90 min at 200 bar. The significantly higher extraction yields obtained using co-solvents at 200 bar, compared to those from pure Sc-CO_2 at 500 bar, highlight the effectiveness of co-solvent addition in enhancing extraction efficiency under milder operating conditions.

Furthermore, by comparing yields of extractible in a single-component (Only CO_2) and binary systems (using ethanol or water as a co-solvent), it is evident that the addition of a polar co-solvent

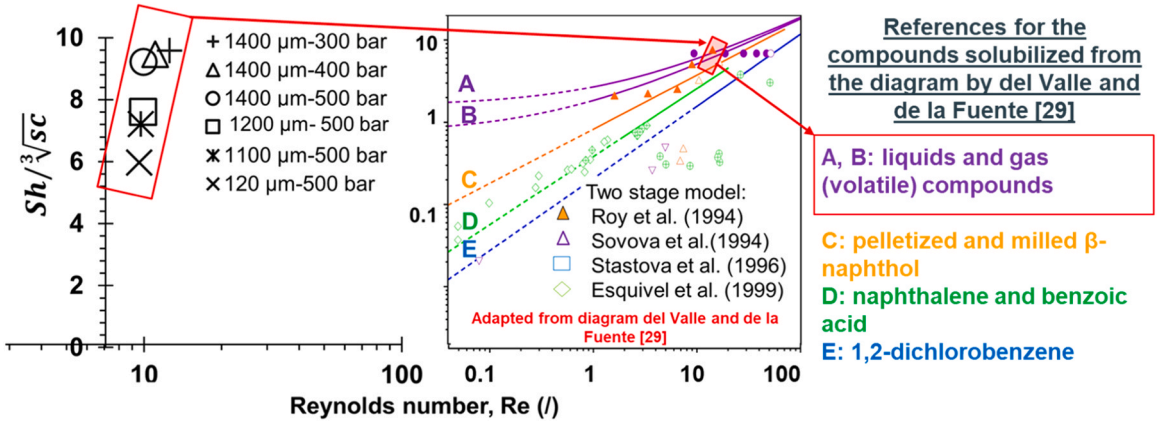


Fig. 5. Influence of Fluid Hydrodynamics on Mass Transfer: Positioning of Our Results Relative to the Literature. (a) External mass transfer results obtained by Cl1 (D[3,4] = 1400 µm) at 300, 400, and 500 bars, and by Cl2 (D[3,4] = 1100 µm), Cl3 (D[3,4] = 120 µm), and the raw (D[3,4] = 1200 µm) at 500 bars. (b) Adapted from Del Valle and de la Fuente (2006) diagram: Dimensionless $Sh/\sqrt[3]{Sc}$ versus Re for literature values of the external mass transfer coefficient in high-pressure oil extraction in a fixed bed from plant substrates. A, B, C, D, and E are overlaid from literature data as reference points for the solubilized components. △: Ground tomato seeds [30]; △: Ground grape seeds [31]; □: Ground sea buckthorn seeds and pulp [32];: ◇: Pre-pressed olive pomace [33].

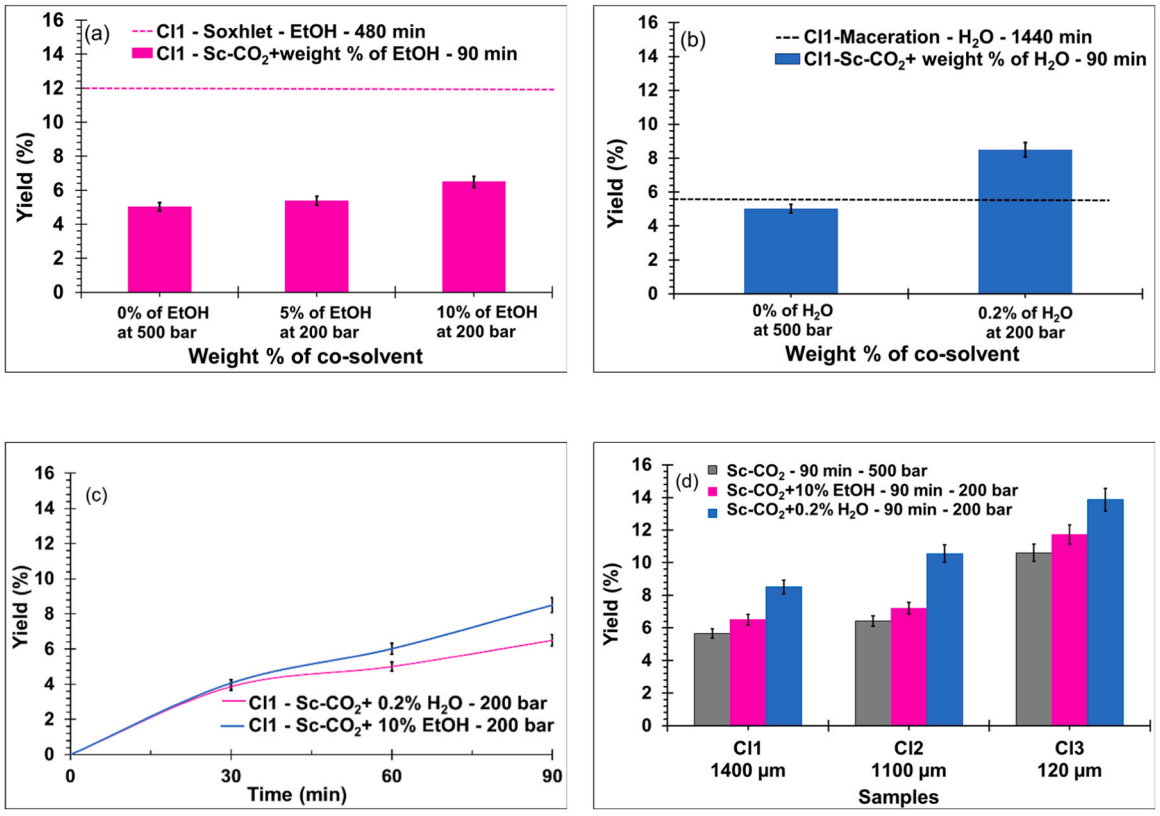


Fig. 6. Effect of co-solvent on extraction yields at 200 bar, 35 °C, and 4 g/min CO_2 flow rate. (a) Weight % of ethanol; (b) Weight % of water; (c) extraction time effect; (d) granulometry effect. The results are expressed as means ± SD (n = 3).

significantly enhances the solubility of polar bioactive compounds. This enhancement arises from the ability of ethanol or water to form hydrogen bonds with the solutes, thereby facilitating their solubility compared to Sc-CO_2 alone. The same trend was also observed by Radzali et al. [37] when comparing the extraction yield of phenolic compounds from *Labisia pumila* using only Sc-CO_2 to a modified technique, where the highest yield of 6.95 % w/w was obtained with polar co-solvents such as ethanol, methanol, and water. Páramos et al. [38] demonstrated that ethanol provided the best extraction results of bioactive compounds from avocado seeds and peels in Sc-CO_2 as solvent/cosolvent.

Furthermore, regardless of the operation conditions, the yields obtained for ethanol-modified extractions were lower than those obtained for water-modified extractions, which could be explained by the higher polarity and shorter molecular structure of water, making it more effective in solute extraction [39].

Comparing the yields obtained using Sc-CO_2 with H_2O and EtOH to conventional techniques such as maceration in water and Soxhlet extraction with ethanol, the yield achieved by Sc-CO_2+ 0.2 % H_2O was higher (8.5 %) than that obtained by maceration in water (5 %) for Cl1.

However, Soxhlet extraction with EtOH yielded a higher yield (12 %) compared to Sc-CO_2+x% EtOH. In comparison to the literature, Soxhlet extraction with ethanol proves to be more efficient and yields higher results. Salim et al. [40] reported a yield of 15.5 % using Soxhlet extraction with ethanol from powdered whole cones of *Pinus halepensis*. Similarly, Albazaz et al. [41] achieved a yield of 16 % using Soxhlet with ethanol from the same plant material.

3.3. Characterization of extracts

The extracts obtained under optimal SFE conditions (Sc-CO_2 at 500 bar, Sc-CO_2 + 10 % EtOH and Sc-CO_2 + 0.2 % H_2O at 200 bar) were characterized for their total polyphenol content, reducing sugar content, antioxidant and anticancer activities, as well as for the identification of non-volatile compounds by HPLC and volatile compounds by GC-MS. The results were compared with those of extracts obtained using conventional methods (Soxhlet extraction with hexane and ethanol, and maceration in water, respectively).

3.3.1. Total polyphenols content

The TPC was determined using the F-C method for the 15 extracts. Fig. 7 illustrates the results obtained after 90 min of extraction using pure Sc-CO_2, Sc-CO_2+ 0.2 % H_2O, and Sc-CO_2+ 10 % EtOH for Cl1, Cl2, Cl3, and raw, in comparison to Soxhlet extraction with hexane, maceration in water, and Soxhlet with ethanol for Cl1, respectively.

The results revealed a significant difference in TPC depending on the extraction techniques and solvents used ($p \leq 0.05$). Pure Sc-CO_2 extraction at 500 bars, 35 °C for 90 min, and a flow rate of 4 g/min resulted in moderate TPC values ranging from 15 to 19 mg GAE/g DW for the raw and different PA-P classes. These values were comparable to those obtained by Soxhlet extraction with hexane for Cl1, suggesting that pure Sc-CO_2 is as effective as Soxhlet with hexane for polyphenol extraction in this matrix. However, it is necessary to use co-solvent, such as ethanol or water, which may enhance the extraction of total polyphenols [26,42].

In this study, the addition of 0.2 % water as a co-solvent to pure Sc-CO_2 extraction resulted in significantly higher TPC values (up to 60 mg GAE/g DW) compared to those obtained with pure Sc-CO_2 and Sc-CO_2+ 10 % EtOH ($p \leq 0.05$). These results suggest that even a small amount of water can enhance the solubility of high molecular-weight polyphenols, which are poorly soluble in pure CO_2 [43]. Water operates as a co-solvent by modifying the polarity of the mixture, thereby promoting the solubilization of more polar compounds, such as polyphenols. Thermodynamically, water lowers the Gibbs free energy of solvation for high molecular-weight polyphenols due to its strong hydrogen-bonding capacity and high cohesive energy [44]. These interactions enhance the solubility of polar compounds that are poorly solvated by pure Sc-CO_2 and Sc-CO_2+ 10 % EtOH. Furthermore, water may enhance extraction efficiency by facilitating the diffusion of extractable compounds such as polyphenols through plant tissues. It can also swell the plant matrix, increasing internal diffusion and promoting the release of bound phenolics. Additionally, water's lower viscosity compared to ethanol may be preferred for preparing plant extract due to the possibility of accelerating mass transfer [45]. Thus, water enhances both solubility and mass transfer, improving extraction efficiency through multiple mechanisms.

Regarding the impact of granulometry, TPC values obtained with Sc-CO_2+ 0.2 % H_2O increased from 50 to 70 mg GAE/g DW as particle size decreased from Cl1 (1400 µm) to Cl2 (1100 µm) and Cl3 (120 µm), confirming the significant effect of particle size on polyphenol extraction ($p \leq 0.05$). Smaller particles increase the contact surface, facilitating the solubilization of polyphenols. The TPC of the raw (unsieved) sample was consistent with its intermediate particle size and aligned with the mass balance, confirming the reliability of the data.

However, TPC values obtained with pure Sc-CO_2 and Sc-CO_2 with co-solvent extraction methods were still significantly lower than those achieved with conventional techniques ($p \leq 0.05$), such as maceration in water, which reached a TPC of 240 mg GAE/g DW for Cl1, six times higher than that obtained with Sc-CO_2+ 0.2 % H_2O. This marked difference underscores the superior efficiency of aqueous maceration for polyphenol extraction, likely due to water's better affinity for these compounds.

It is important to note that these analyses are being conducted for the first time on *P. halepensis* petals, with no prior studies available, making comparisons with the literature challenging. Compared to the literature, Nuralın et al. [19] reported that adding 2–8 % w/w ethanol as a co-solvent significantly increased polyphenol recovery (e.g., quercetin, rutin, kaempferol) from 6.03 to 44.68 µg/g in *P. brutia* pinecones under 120 min, aligning with our findings. Da Porto et al. [46] used Sc-CO_2 with water and ethanol as co-solvents to extract polyphenols from grape clusters, observing that adding water resulted in a higher TPC, which is consistent with our results. Furthermore, Chupin et al. [47] demonstrated the significant impact of particle size on TPC using ethanol-water extraction of maritime pine bark with microwave-assisted techniques, reporting the highest concentration (39.52 mg GAE/g DW) for finer particles, which is consistent with our results. Páramos et al. [38] demonstrated that ethanol provided the best TPC of bioactive compounds from avocado seeds and peels in Sc-CO_2 as solvent/cosolvent.

3.3.2. Reducing sugar content

The RSC was determined using the DNSA method for the 15 extracts. Fig. 8 illustrates the results obtained after 90 min of extraction using pure Sc-CO_2, Sc-CO_2+ 0.2 % H_2O, and Sc-CO_2+ 10 % EtOH for Cl1, Cl2, Cl3, and raw, in comparison to Soxhlet extraction with hexane,

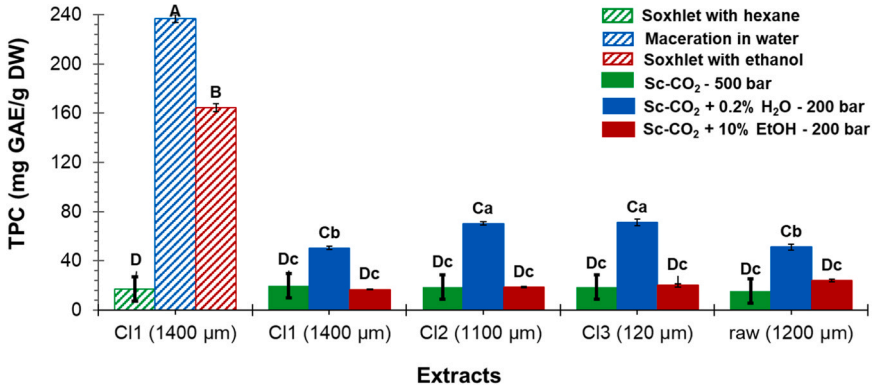


Fig. 7. The total phenolic content (TPC) of the extracts from the petals of P. halepensis. Each letter on the table represents a significant difference ($p \leq 0.05$). Uppercase and lowercase letters mean a significant difference in extraction techniques and granulometry, respectively. The results are expressed as means ± SD (n = 3).

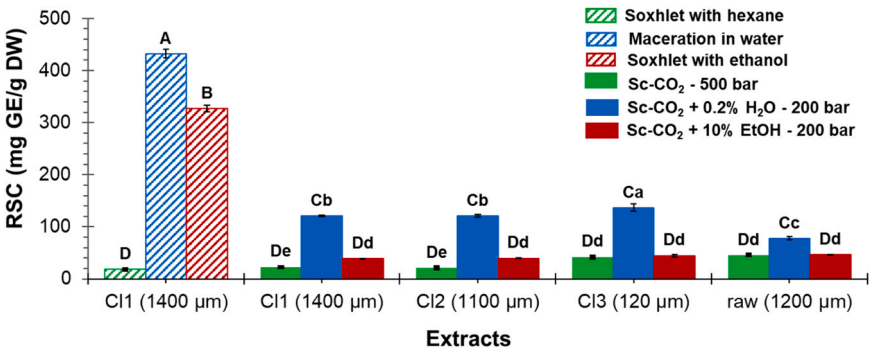


Fig. 8. The reducing sugar content (RSC) of the extracts from the petals of P. halepensis. Uppercase and lowercase letters mean a significant difference in extraction techniques and granulometry, respectively. The results are expressed as means ± SD (n = 3).

maceration in water, and Soxhlet with ethanol for Cl1, respectively.

Extraction with pure Sc-CO_2, performed at 500 bar, 35°C for 90 min with a flow rate of 4 g/min, yielded moderate reducing sugar contents ranging from 21 to 42 mg GE/g DW for the different PA-P classes (Cl1, Cl2, Cl3, and raw). The highest value was obtained for the Cl3, highlighting the significant impact of particle size on reducing sugar extraction ($p \leq 0.05$). However, these concentrations remain relatively low, suggesting that pure Sc-CO_2 is not optimal for extracting reducing sugars.

The addition of 0.2 % water as a co-solvent in Sc-CO_2 extraction resulted in significantly higher reducing sugar contents compared to pure Sc-CO_2 and Sc-CO_2+ 10 % EtOH ($p \leq 0.05$). The RSC obtained with Sc-CO_2+ 0.2 % H_2O increased from 78 to 120 mg GE/g DW from Cl1 to Cl3, highlighting the significant impact of particle size on sugar extraction ($p \leq 0.05$). Furthermore, even small amounts of water enhance reducing sugar solubility. However, RSC values obtained with Sc-CO_2 and co-solvents were significantly lower than those achieved by maceration in water and Soxhlet ethanol extraction, which yielded 432.12 and 327.13 mg GE/g DW for Cl1, respectively ($p \leq 0.05$).

These analyses are being conducted on PA-P for the first time, and no similar prior studies exist, making it difficult to compare our findings with the literature. Chupin et al. [47] quantified total sugars in extracts from varying particle sizes of maritime pine bark (*Pinus pinaster*) using microwave-assisted extraction, highlighting a significant impact of granulometry on sugar content. Higher concentrations were observed in fine particles (4.53 mg GE/g matrix), aligning with our findings.

3.3.3. Antioxidant activity

The AOA was evaluated for the 15 extracts obtained from raw and different PA-P classes (Cl1, Cl2, Cl3) against DPPH. Extracts were assessed at 50 µg/mL. Vitamin C at 4 g/L was used as a reference.

The results reveal a significant difference in the inhibition of DPPH by the extracts, depending on the methods and solvents used ($p \leq 0.05$). For SFE, only the extracts obtained with Sc-CO_2+ 0.2 % H_2O showed interesting inhibition percentages (30–60 %). However, water maceration and Soxhlet extraction with EtOH resulted in higher inhibition percentages (greater than 80 %). These results correlate with the polyphenol content. Polar solvents, particularly water and ethanol, proved to be effective solvent systems for extracting total phenolic compounds, highlighting the importance of phenolic compounds as natural antioxidants in enhancing free radical scavenging activity [48].

To assess the effectiveness of the extracts with significant inhibition (above 50 %) in their antioxidant activity, IC_{50} was determined from the calibration curve. A lower IC_{50} value indicates higher antioxidant potential. As presented in Table 3, for Sc-CO_2+ 0.2 % H_2O extracts, Cl3 (120 µm) shows the lowest IC_{50} (37.57 µg/mL), indicating the significant impact of granulometry on antioxidant activity ($p \leq 0.05$). However, this antioxidant potential remains significantly lower compared to extracts from maceration in water (11.39 µg/mL) and Soxhlet extraction with ethanol (13.11 µg/mL) ($p \leq 0.05$), suggesting that conventional extraction techniques provide higher-quality extracts than SFE extraction.

Table 3
IC_{50} of the antioxidant activity of extracts from P. halepensis petals.

PA-P extracts	DPPH IC_{50} (µg/mL)
Cl1 (1400 µm) – Soxhlet with hexane	Na
Cl1 (1400 µm) – pure Sc-CO_2	Na
Cl2 (1100 µm) – pure Sc-CO_2	Na
Cl3 (120 µm) – pure Sc-CO_2	Na
raw (1200 µm) – pure Sc-CO_2	Na
Cl1 (1400 µm) – maceration in water	11.39 ± 0.57^{d}
Cl1 (1400 µm) – Sc-CO_2+ 0.2 % H_2O	59.22 ± 1.53^{a}
Cl2 (1100 µm) –Sc-CO_2+ 0.2 % H_2O	46.95 ± 4.21^{b}
Cl3 (120 µm) – Sc-CO_2+ 0.2 % H_2O	36.36 ± 2.59^{c}
raw (1200 µm) – Sc-CO_2+ 0.2 % H_2O	37.57 ± 0.01^{c}
Cl1 (1400 µm) – Soxhlet-EtOH	13.11 ± 0.05^{d}
Cl1 (1400 µm) – Sc-CO_2+ 10 % EtOH	Na
Cl2 (1100 µm) – Sc-CO_2+ 10 % EtOH	Na
Cl3 (120 µm) – Sc-CO_2+ 10 % EtOH	Na
raw (1200 µm) – Sc-CO_2+ 10 % EtOH	Na
Vitamin C	3.06 ± 0.09

PA-P: P. halepensis petals; raw, Cl1, Cl2 et Cl3 at mean diameter of particle D [3,4]: 1200, 1400, 1100, 120 µm respectively; Sc-CO2: Supercritical carbon dioxide; EtOH: ethanol; IC50: half-maximal inhibitory concentration; Vitamin C: reference. na: non active. Each letter (a–d) on the table represents a significant difference ($p \leq 0.05$). The results are expressed as means ± SD (n = 3).

These analyses are being conducted on PA-P for the first time, and no similar prior studies exist, making it difficult to compare our findings with literature. Gião et al. [49] showed that particle size significantly affects antioxidant capacity, with the highest activity observed in *Agrimonia eupatoria* extracts from particles smaller than 0.2 mm, which aligns with our results. Similarly, Salim et al. [40] found that maceration extraction of *P. halepensis* pinecones yielded a more favorable IC_{50} (1.4 µg/mL) compared to Soxhlet extraction with ethanol (3.2 µg/mL), which follows the same trend observed in our findings.

3.3.4. Anticancer activity

The results of ACA were reported in Table 4 presenting the inhibition percentages of the extracts against two cancer cell lines, LS174t (female-type human colon adenocarcinoma) and HCT116 (male-type human colon adenocarcinoma), as well as the normal human embryonic kidney cell line (HEK293) for the different extracts obtained by maceration, Soxhlet and Sc-CO_2 with various solvents (hexane, water, ethanol) at different particle sizes. Extracts were assessed the anticancer activity at 50 µg/mL. Tamoxifen was used as a reference at 100, 10, and 1 µM.

All extracts demonstrated moderate to high anticancer activity against the LS174t and HCT116 cell lines compared to tamoxifen (well-

Table 4
Viability inhibition by P. halepensis petal extracts in cancer and normal cell lines.

Extracts	% Inhibition LS174t	% Inhibition HCT116	% Inhibition HEK293
Cl1 (1400 μm) – Soxhlet with hexane	27.90 ± 4.86^{b}	36.29 ± 1.28^{a}	33.42 ± 3.83^{a}
Cl1 (1400 μm) – pure Sc-CO_2	7.13 ± 2.77^{e}	25.95 ± 3.44^{b}	14.69 ± 2.09^{c}
Cl2 (1100 μm) – pure Sc-CO_2	14.97 ± 2.31^{d}	34.25 ± 2.13^{a}	19.02 ± 1.71^{b}
Cl3 (120 μm) – pure Sc-CO_2	24.45 ± 2.75^{c}	37.53 ± 2.67^{a}	21.34 ± 0.45^{b}
raw (1200 μm) – pure Sc-CO_2	5.18 ± 0.03^{e}	21.66 ± 2.28^{b}	20.29 ± 6.62^{b}
Cl1 (1400 μm) – maceration in water	60.49 ± 1.87^{a}	16.30 ± 2.45^{c}	8.07 ± 1.51^{c}
Cl1 (1400 μm) – Sc-CO_2+ 0.2 % H_2O	24.69 ± 5.01^{b}	29.50 ± 1.38^{b}	7.92 ± 1.14^{c}
Cl2 (1100 μm) –Sc-CO_2+ 0.2 % H_2O	33.48 ± 5.80^{b}	36.09 ± 3.66^{a}	13.08 ± 0.73^{c}
Cl3 (120 μm) – Sc-CO_2+ 0.2 % H_2O	36.36 ± 2.59^{b}	35.57 ± 4.16^{a}	10.25 ± 3.27^{c}
raw (1200 μm) – Sc-CO_2+ 0.2 % H_2O	13.95 ± 0.20^{d}	30.11 ± 0.91^{a}	5.24 ± 2.10^{c}
Cl1 (1400 μm) – Soxhlet-EtOH	1.39 ± 0.08^{e}	21.86 ± 4.42^{b}	22.09 ± 10.40^{b}
Cl1 (1400 μm) – Sc-CO_2+ 10 % EtOH	24.53 ± 5.57^{b}	32.44 ± 6.16^{a}	23.17 ± 4.20^{b}
Cl2 (1100 μm) – Sc-CO_2+ 10 % EtOH	24.81 ± 1.67^{c}	35.40 ± 2.44^{a}	12.01 ± 3.35^{c}
Cl3 (120 μm) – Sc-CO_2+ 10 % EtOH	28.29 ± 4.84^{b}	42.58 ± 2.19^{a}	11.13 ± 4.60^{c}
raw (1200 μm) – Sc-CO_2+ 10 % EtOH	24.49 ± 3.14^{b}	35.17 ± 2.26^{a}	19.31 ± 4.94^{b}
tamoxifen	59.89 ± 4.12	70.33 ± 3.91	68.63 ± 2.46

raw, Cl1, Cl2, and Cl3 at D[3,4]: 1200, 1400, 1100, 120 μm, respectively; Sc-CO2: supercritical carbon dioxide; EtOH: ethanol; LS174t cell line: female-type human colon adenocarcinoma; HCT116 cell line: male-type human colon adenocarcinoma; HEK293 cell line: human embryonic kidney cell line; extract were assessed at 50 μg/mL. tamoxifen at 100 μM: reference. Each letter (a–e) on the table represents a significant difference ($p \leq 0.05$). Results are expressed as mean ± SD (n = 3).

known anticancer standard). The highest inhibition was recorded for the Cl1 extract obtained via maceration in water (60.49 %) against LS174t and the Cl3 extract obtained via Sc-CO_2+ 10 % EtOH (42.58 %) against HCT116 ($p \leq 0.05$). Additionally, all extracts showed low to moderate inhibition (ranging from 5 % to 30 %) against the normal HEK293 cell line, indicating the extracts' low toxicity.

These analyses were conducted for the first time, making bibliographic comparisons unavailable. In the study by Gascón et al. [50], the Caco-2 cell line, derived from colon cancer, was used to evaluate *P. halepensis* bark extracts, revealing an inhibitory effect ranging from 25 % to 60 %. This aligns with our findings and confirms that *P. halepensis* exhibits moderate to high anticancer potential against colon cancer. Additionally, the essential oils from seeds and cones of *Abies concolor* (*Pinaceae*) showed no toxicity toward normal human cells, such as skin fibroblasts and endothelial cells [51]. This supports the low in vitro cytotoxicity of *Pinaceae* cone extracts, suggesting their potential for cosmetic and pharmaceutical applications, pending further comprehensive toxicological evaluation.

3.3.5. Identification of "non-volatile" compounds

The HPLC-DAD analysis was conducted for the 15 extracts (raw and the different PA-P classes: Cl1, Cl2, Cl3) using pure Sc-CO_2, Sc-CO_2+ 0.2 % H_2O, and Sc-CO_2+ 10 % EtOH, compared to Soxhlet extraction with hexane, maceration in water, and Soxhlet extraction with ethanol, respectively, as reported in Table 5.

The composition of the extracts was identified by comparing the retention time and maximum wavelength of each peak with those of reference compounds injected under the same conditions. This analysis led to the identification of 38 molecules, 24 of which were common across all extracts, with 33 identified in Sc-CO_2+ 0.2 % H_2O extracts, 32 in pure Sc-CO_2 extracts, and 30 in Sc-CO_2+ 10 % EtOH extracts.

The effect of extraction techniques was evaluated for Cl1 extracts, while the impact of granulometry was assessed on the different classes and the raw material extracts using pure and modified Sc-CO_2. The results revealed a significant difference. Soxhlet extraction with hexane was the most effective method for extracting certain moderate and non-polar compounds, yielding higher peak areas for compounds such as chrysine (17.35), 7-hydroxyflavone (40.12), shikonin (21.12), 3′-hydroxy-6-methylflavone (13.08), and 7-hydroxy-3′,4′,5′-trimethoxy-alpha-naphthoflavone (15.09). However, pure Sc-CO_2 demonstrated superior efficiency to Soxhlet with hexane for catechin, 3′-hydroxy-alpha-naphthoflavone, and rosmarinic acid, suggesting a higher affinity of these compounds for supercritical CO_2. Adding 10 % ethanol or 0.2 % water significantly enhanced the extraction of polar compounds. Catechin increased from 3.16 (Sc-CO_2, Cl2) to 9.08 with 0.2 % H_2O and 5-hydroxyflavone rose from 21.26 to 43.43 (Cl3). This highlights the role of co-solvents in improving bioactive compound extraction. Granulometry plays a critical role in extraction efficiency. Finer particle extracts (Cl3) present more bioactive compounds than coarser ones. For instance, trihydroxyethylrutin showed significantly higher peak areas in Cl3 (19.91) compared to Cl1 (8.72). Similarly, chlorogenic acid increased from 9.09 in Cl1 to 59.59 in Cl3, attributed to the increased surface area in finer particles, enhancing solvent-target compound interaction. The maceration in water and Sc-CO_2+ 0.2 % H_2O extracts, which exhibit significant antioxidant and anticancer potential, are rich in polar compounds such as caffeic acid, trihydroxyethylrutin, chlorogenic acid, gallic acid, and beta-carotene. These compounds were well-known in scientific research for their biological activities. Caffeic and chlorogenic acids were particularly recognized for their powerful antioxidant properties, which neutralize free radicals and reduce oxidative stress [52]. They also showed promise as photoprotective agents, incorporated into skincare products due to their antioxidant activity [53]. Additionally, Caffeic and chlorogenic acids exhibited anticancer properties by inhibiting tumor cell proliferation, inducing apoptosis, and suppressing angiogenesis and metastasis [54], suggesting that these molecules contribute to the anticancer activity observed in this study. Trihydroxyethylrutin was recognized in pharmacology for its antioxidant and anticancer properties, providing vasculoprotective and anti-inflammatory benefits [55]. Gallic acid demonstrated antioxidant and antimicrobial properties, protecting against oxidative damage and inflammation while promoting apoptosis in cancer cells [56]. Finally, beta-carotene was known as an antioxidant, shielding cells from oxidative damage and contributing to the prevention of chronic diseases, including cancer and cardiovascular diseases [57]. Based on these findings, it was reasonably deduced that these molecules were responsible for the notable biological activities observed in the aqueous extracts studied.

3.3.6. Identification of "volatile" compounds

The GC-MS analysis was conducted for the 15 extracts (raw and from different PA-P classes (Cl1, Cl2, Cl3)) using pure Sc-CO_2, Sc-CO_2+ 0.2 % water, and Sc-CO_2+ 10 % EtOH, compared to extraction by Soxhlet with hexane, maceration in water, and Soxhlet with ethanol, respectively (Table 6).

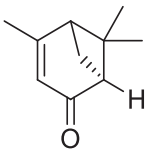
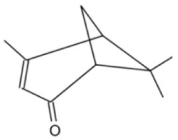
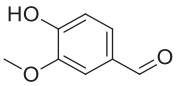
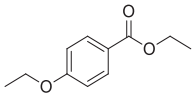
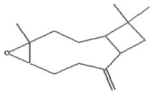
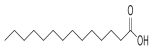
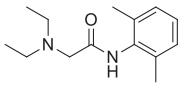
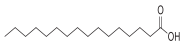
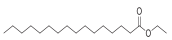
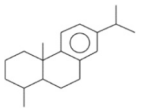
Twenty-four molecules were identified prior to derivatization, including seven compounds were detected for the first time in *P. halepensis*: ethyl 4-ethoxybenzoate, ethyl hexadecanoate, podocarpa-8,11,13-triene-7β,13-diol, 14-isopropyl-, dehydroabietyl alcohol, methyl (1S,4aS,9 R,10aR)-7-isopropyl-9-methoxy-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene, methyl 15-hydroxy-dehydroabietate, and methyl 1-phenanthrene-1-carboxylate,1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-9-oxo-.

Table 5
Identification of "Non-Volatile" organic compounds by HPLC-DAD from Extracts of P. halepensis Petals.

Compounds	RT min	Peak area (mAU*min)															References
		Extracts															
		Soxhlethexane	Pure Sc-CO_2				Soxhlet EtOH	Sc-CO2+ 10 % EtOH				Mace-ration water	Sc-CO_2+ 0.2 % H_2O				
		Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	
trihydroxyethylrutin	0.86	-	-	-	-	-	-	-	-	-	-	25.01	8.72	13.61	19.91	26.75	[51]
catechin	0.90	2.41	1.27	3.16	9.71	4.69	47.53	3.20	1.48	28.69	2.35	20.92	1.59	9.08	13.52	44.02	[54,58]
2,4-dihydroxycinnamic acid	0.93	-	-	-	26.95	-	-	-	-	19.63	-	-	-	-	-	-	
ellagic acid	0.96	-	-	-	11.18	-	-	-	11.95	-	15.82	-	-	-	-	-	
(+-) synephrin	0.99	22.20	22.56	-	-	-	-	-	-	-	-	87.60	20.88	-	43.72	-	
Chlorogenic acid	1.19	-	-	-	-	-	-	-	-	-	-	66.21	9.09	30.04	59.59	-	
Gallic acid	2.05	-	-	-	-	-	-	-	-	-	-	59.65	-	-	52.06	-	
3,4-dihydroxycinnamic acid	3.15	-	-	-	-	-	-	-	-	-	-	3.32	-	-	1.61	-	
6-hydroxyflavone	15.58	-	-	-	8.64	-	-	-	-	-	-	-	-	-	-	-	
5-hydroxy−4′-methoxylflavone	18.70	-	21.11	-	-	-	-	-	-	-	-	-	-	-	-	-	
chrysin	18.92	17.35	0.04	-	-	0.04	-	-	-	-	-	-	-	-	-	-	
warfarin ou 4-hydroxy−3-(3-oxo−1-phenylbutyl)coumarine	19.10	-	-	-	0.04	-	-	-	-	-	0.04	-	0.13	10.43	-	42.66	
3'-hydroxy-a-naphthoflavone	19.37	-	-	36.11	0.12	50.10	-	41.54	-	-	-	-	-	-	47.38	-	
3-tert-butyl−4-hydroxybenzoic acid	19.44	-	-	-	44.67	10.14	-	-	0.60	-	62.20	-	13.70	-	64.67	-	
5,7-dihydroxy−3′,4′,5′-trimethoxyflavone	19.52	-	-	13.79	-	-	77.91	12.36	-	63.87	-	-	17.69	15.38	-	40.35	
7-hydroxyflavone	19.70	40.12	12.69	19.20	51.08	11.04	25.39	16.26	-	-	22.14	3.22	-	-	17.02	18.78	
beta carotene	19.79	-	-	-	-	-	-	-	-	21.08	-	-	22.95	11.93	-	-	
luteine	19.81	-	36.68	12.07	10.11	20.39	15.82	-	55.78	41.06	15.86	4.08	12.21	-	10.46	28.50	
5,7-dihydroxy−4-propylcoumarine	20.62	14.57	9.69	14.72	19.89	9.97	13.02	-	7.59	10.38	15.59	0.18	9.81	11.45	17.31	16.62	
shikonin	20.70	21.12	7.95	-	11.25	9.94	7.18	10.18	-	-	-	-	-	-	9.45	15.13	
3′-hydroxy−6-methylflavone	20.75	13.08	7.13	13.19	12.77	14.21	16.48	-	24.04	20.39	11.78	-	14.50	3.13	-	-	
5-hydroxyflavone	21.00	31.36	18.36	20.81	21.26	17.01	26.72	6.81	-	-	22.84	-	22.28	13.08	43.43	29.71	
diethylstilbestrol	21.09	-	-	-	10.91	-	-	17.06	13.20	-	-	-	-	-	-	-	
butyle 4-hydroxybenzoate	21.12	23.43	22.03	-	13.53	12.35	12.86	-	20.60	-	19.21	0.66	12.90	17.80	28.79	24.22	
cardamonin	21.26	-	23.30	18.86	15.98	10.70	25.71	13.51	11.44	12.09	20.49	-	16.99	14.49	16.91	19.37	
cafeic acid 1,1-dimethylallyl ester	21.34	25.78	22.15	-	-	-	-	-	14.95	20.99	-	-	-	11.17	26.78	-	
4-hydroxy−3-propylbenzoate de méthyle	21.42	-	-	-	-	-	9.90	-	-	15.18	-	-	-	20.98	18.22	18.34	
7-hydroxy−3′,4′,5′-trimethoxy-alpha-naphthoflavone	21.50	15.19	12.68	18.49	37.76	8.07	14.13	17.25	16.05	0.14	16.27	17.56	15.58	6.37	21.06	10.86	
3,3′-dimethoxyflavone	21.60	-	41.17	22.82	-	16.55	25.56	-	41.00	0.51	35.90	27.33	-	22.94	39.92	31.94	
3,6,3′-trimethoxyflavone	21.78	-	40.67	28.01	-	18.71	22.49	24.39	25.73	28.36	29.27	0.72	23.75	20.37	27.81	21.87	
3,7-dimethoxyflavone	21.83	25.99	-	36.92	34.14	25.42	14.77	-	36.41	38.44	39.87	-	19.45	24.18	35.85	35.91	
5-hydroxy−3′-methoxyflavone	22.01	40.89	12.80	12.89	44.72	13.13	19.97	36.70	-	13.75	14.24	0.30	8.78	13.69	34.76	16.06	
xanthurenic acid	22.12	13.30	15.36	17.94	21.00	13.65	9.37	19.86	15.30	-	-	-	4.20	10.04	15.40	7.02	
4',5′-dimethoxy−2′-hydroxy−4-methylchalcone	22.60	10.35	15.23	19.96	31.51	-	20.18	32.83	-	-	-	-	19.84	13.06	33.69	14.62	
rosmarinic acid	22.67	21.82	46.27	-	-	-	-	-	23.53	18.40	-	0.59	8.61	8.85	-	11.99	
(z)−3-(3-ethoxy−4-hydroxy-phenyl)−2-phenyl-acrylic acid	23.40	23.32	0.54	-	-	15.38	60.57	11.34	-	-	50.31	-	21.15	1.28	-	-	
hamamélitannin	24.06	15.54	7.82	32.01	16.84	17.91	46.20	4.97	20.04	35.37	32.49	15.24	15.02	23.92	41.11	33.55	
3,4-dihydroxy−5-methoxycinnamic acid	25.04	-	16.16	4.97	3.21	9.45	-	-	5.13	3.38	-	-	-	-	-	-	

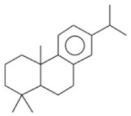
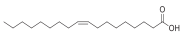
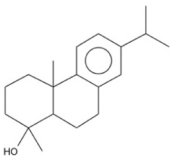
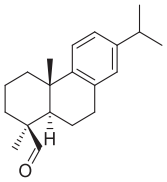
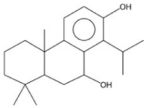
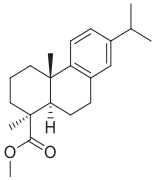
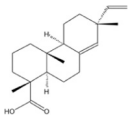
raw, Cl1, Cl2 et Cl3 at D[3,4]: 1200, 1400, 1100, 120 μm respectively; Sc-CO2: Supercritical carbon dioxide; EtOH: ethanol; "-": not detected.

Table 6
Identification of compounds by GC-MS.

N°	Compounds	RT min	Structure	Peak area × 10^{07}															Ref.
				Soxhlet hexane	Pure Sc-CO_2				Soxhlet EtOH	Sc-CO_2 + 10 % EtOH				Maceration water	Sc-CO_2 + 0.2 % H_2O				
				Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	
1	Bicyclo[3.1.1]hept−3-en−2-one, 4,6,6-trimethyl-, (1S)-	7.81		-	-	-	-	-	-	-	-	-	-	-	-	-	-	58	[62]
2	D-verbenone	7.84		16.7	2.11	17.2	73.7	-	10.8	-	7.81	-	-	-	-	23.2	-	-	[63]
3	Vanillin	10.37		-	-	-	-	-	-	-	-	-	-	-	19.5	-	-	-	[64]
4	ethyl 4-éthoxybenzoate	12.15		-	-	-	-	-	8.37	-	-	-	-	-	8.18	6.07	1.49	-	[65]
5	Caryophyllene oxide	12.96		75.3	18.4	21.1	90.6	7.53	-	16.6	61.5	20.9	21.3	-	18.8	46	8.51	51	[59]
6	myristic acid	14.85		-	-	-	-	20.5	-	14.6	-	-	-	-	-	-	2.99	-	[66]
7	Lidocaïn	16.22		-	-	-	-	-	35	33.5	24.8	47.5	52.3	-	30.9	-	5.86	-	[67]
8	Palmitic acid	16.77		14	50.2	49.8	67.4	55.3	260	129	133	144	92.4	-	37.7	133	6.16	-	[68]
9	Ethyl hexadecanoate	16.96		-	26.5	-	-	19.2	-	27.7	-	18.3	13.4	-	5.23	13.8	2.1	-	[69]
10	18-Norabieta−8,11,13-triene	17.4		82.3	22.2	52.1	129	17.2	23.3	43	95.3	52.3	61.4	-	23.6	72.2	21.8	4.73	[70]

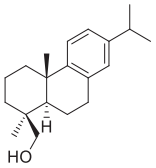
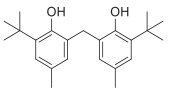
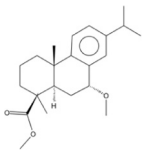
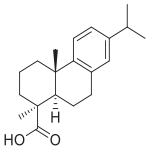
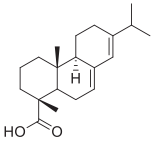
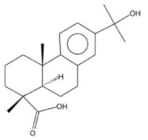
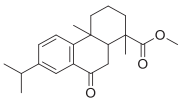
(*continued on next page*)

Table 6 (*continued*)

N°	Compounds	RT min	Structure	Peak area × 10^{07}															Ref.
				Soxhlet hexane	Pure Sc-CO_2				Soxhlet EtOH	Sc-CO_2 + 10 % EtOH				Mace-ration water	Sc-CO_2 + 0.2 % H_2O				
				Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	
11	Dehydroabietane	17.69		41.6	12.1	29.2	66.2	12.5	12.4	24.7	52.6	26.3	31.8	-	12.5	43.3	10.4	24.5	[71]
12	Oleic acid	18.24		-	-	34.3	-	-	-	-	-	-	-	-	13.2	40.5	-	-	[72]
13	7-Isopropyl−1,4a-dimethyl−1,2,3,4,4a,9,10,10a-octahydro−1-phenanthrenol (isomer 1)	18.8		49.2	28.9	432	755	196	545	106	169	142	139	-	17.0	15.7	39.8	104	
14	Dehydroabietal	19.42		-	-	-	-	-	146	39	-	-	383	-	201	405	563	409	
15	Podocarpa−8,11,13-triene−7β,13-diol, 14-isopropyl-	19.66		-	30.3	-	121	15.5	15.7	30.2	-	-	48.6	-	15.8	71.3	13.5	31.1	
16	Methyl dehydroabietate	19.85		74.8	47.2	44.6	83.5	25.7	24.8	51.1	71.7	41.5	53.7	-	38.6	63.1	17.8	37.1	[73]
17	Cryptopimaric acid	20.09		-	-	-	-	-	17.2	-	-	-	93.2	-	27.5	88.6	10.6	-	[60]

(*continued on next page*)

Table 6 (*continued*)

N°	Compounds	RT min	Structure	Peak area × 10^{07}															Ref.
				Soxhlet hexane	Pure Sc-CO_2				Soxhlet EtOH	Sc-CO_2 + 10 % EtOH				Mace-ration water	Sc-CO_2 + 0.2 % H_2O				
				Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	Cl1	Cl1	Cl2	Cl3	raw	
18	Dehydroabietyl alcohol	20.15		74.9	48.4	59.3	116	33.2	38.6	52.3	96.5	91.8	57.3	-	23.1	63.3	23.8	41.5	[61]
19	Phenol, 2,2′-méthylènobis[6-(1,1-diméthylethyl)−4-méthyl-	20.32		97.4	-	389	126	56.5	143	207	101	72.9	115	2.38	18.3	317	-	20.1	
20	Methyle (1S,4aS,9 R,10aR)−7-isopropyl−9-méthoxy−1,4a-diméthyl−1,2,3,4,4a,9,10,10a-octahydrophénanthrène	20.55		34	25.2	-	-	-	-	23.1	33.1	192	221	-	-	29.6	8.19	27.4	
21	Dehydroabietic acid	20.76		275	202	296	273	149	122	244	284	289	295	-	128	293	388	242	[59]
22	Abietic acid	21.09		-	-	-	-	-	40.0	54.1	38.8	-	-	-	-	-	8.56	70.7	[59]
23	Methyl ester of 15-hydroxy-dehydroabietic acid	21.39		-	-	-	-	-	-	33	60.5	40.5	41.3	-	21.9	48.5	7.58	36.5	[74]
24	Methyl ester of 1-phenanthrene−1-carboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro−1,4a-dimethyl−7-(1-methylethyl)−9-oxo-	21.86		56.6	-	53	88.9	27.1	13.9	43	73.2	59.8	59.6	-	32.2	60.1	11.1	52	[75]

raw, Cl1, Cl2 et Cl3 at D[3,4]: 1200, 1400, 1100, 120 μm respectively; Sc-CO2: Supercritical carbon dioxide; EtOH: ethanol; Ref.: references; "-": not detected.

The results showed that extraction with pure Sc-CO_2 exhibits a similar efficiency to Soxhlet extraction with hexane for extracting several bioactive compounds, including D-verbenone, caryophyllene oxide, 18-Norabieta-8,11,13-triene, dehydroabietane, methyl dehydroabietate, and dehydroabietyl alcohol. The addition of 10 % ethanol and 0.2 % water to Sc-CO_2 significantly improved the concentration of bioactive compounds, including vanillin, lidocaine, palmitic acid, dehydroabietal, cryptopimaric acid, abietic acid, and methyl 15-hydroxy-dehydroabietate. This suggests that the polar co-solvent enhances the extraction of polar compounds by increasing their solubility in supercritical CO_2. A significant effect of particle size on the composition of the extracts was also observed. The results show that reducing particle size significantly increases bioactive compounds' concentration (surface area). For example, the amount (expressed as unitless peak area, proportional to concentration) of dehydroabietic acid increased from 128 to 289 $\times$ 10^7 and from 244 to 388 $\times$ 10^7 when the particle size was reduced from Cl1 (1400 μm) to Cl3 (120 μm) for Sc-CO_2+ 10 % EtOH and Sc-CO_2+ 0.2 % H_2O, respectively.

Dehydroabietic acid, abietic acid, and methyl 15-hydroxy-dehydroabietate were previously detected in the cones of *P. halepensis, P. brutia,* and *P. pinea* using GC-MS by Kilic et al. [59] and are well-known for their anticancer properties, suggesting that these molecules could be responsible for anticancer activity observed in this study. Similarly, cryptopimaric acid, previously identified in pine species, is recognized for its lipoxygenase inhibitory, antineoplastic, and anticancer activities [60]. Based on these findings, it can be inferred that these compounds are likely responsible for the significant anticancer activities observed in the studied extracts. Furthermore, dehydroabietyl alcohol is widely recognized and used as an ingredient in cosmetics [61], which opens up perspectives for the application of our extracts in the pharmaceutical and cosmetic industries.

4. Conclusion

This study presents a novel and original contribution to biomass valorization through the application of supercritical CO_2 (Sc-CO_2) for the first time to extract bioactive compounds from *P. halepensis* petals. The innovative aspect of this research lies in the identification of seven chemical compounds never previously reported in this species, expanding the chemical knowledge of the Pinus genus.

The effects of extraction parameters, including time, pressure, and granulometry, on the supercritical fluid extraction (SFE) process were investigated. Conventional extraction techniques, such as Soxhlet extraction and maceration, were employed to compare yields and extract quality with those obtained using Sc-CO_2 and modified Sc-CO_2. Extraction kinetics revealed that beyond 20 min, pressure and granulometry have a significant impact on extraction yield. The highest extraction yield was achieved with the smallest particle size using Sc-CO_2 at 500 bar and 35°C after 120 min.

Although Sc-CO_2 offered superior yields in shorter extraction times, conventional methods such as maceration and Soxhlet produced extracts with higher bioactivities. A total of 38 molecules were identified via HPLC-DAD and 24 via GC-MS, highlighting the chemical richness of the extracts.

Based on these findings, which confirm the technical feasibility of Sc-CO_2 extraction, future studies will focus on comprehensive sustainability assessments, such as life cycle analysis (LCA), to quantify the environmental and economic impacts. These evaluations will be essential for positioning this work within a broader framework of circular bioeconomy and sustainable innovation.

CRediT authorship contribution statement

Jalloul Bouajila: Writing – review & editing, Validation, Supervision, Methodology. **Luc Fillaudeau:** Writing – review & editing, Validation, Supervision, Project administration, Methodology. **Irina Smirnova:** Writing – review & editing, Validation, Methodology. **Carsten Zetzl:** Writing – review & editing, Validation, Supervision, Project administration. **Mehrez Romdhane:** Writing – review & editing, Validation, Supervision, Project administration, Methodology. **Amel Chammam:** Writing – original draft, Formal analysis, Data curation.

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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.

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Data availability

The data that has been used is confidential.

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