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Research article

Green strategies for the valorization of industrial medicinal residues of *Serenoa repens* small (saw palmetto) as source of bioactive compounds

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ABSTRACT

Serenoa repens is a medicinal plant well-known for its therapeutic potential in treating various urological disorders and prevention of prostatic cancer. However, the extraction process in the pharmaceutical industry leads to the generation of plant residues, typically discarded, wasting valuable resources. In this study, we aimed to explore a series of green extraction strategies to effectively valorize the residues of *Serenoa repens* fruits. Initially, we employed supercritical CO_2 (1.2% yield on dry biomass) on the discarded biomass to identify and quantify residual fatty acids and polyprenols (1.6% of the extract dry weight), a class of unsaturated isoprenoid alcohols with promising biomedical applications. Subsequently, subcritical water extraction was utilized on the exhausted biomass to extract polar compounds. An increase in the extraction yield was observed with the rise in processing temperature up to 180 °C (yields were found higher than 26%). Phenolic compounds and carbohydrate macromolecules profiles were affected by the increased hydrolytic conditions. Polar extracts exhibited robust bioactivities, demonstrating significant antioxidant activity and antimicrobial efficacy against Gram-positive and Gram-negative bacteria strains. Extracts obtained at 180 °C demonstrated the highest efficacy. Furthermore, *in vitro* assessment of mannans-rich fraction provided a new perspective of potential applications in the cosmeceuticals field. Results underscore the potential of the sustainable extraction biorefinery for the residue of this medicinal plant and demonstrate that, harnessing these bioactive compounds, new sustainable and eco-friendly approaches for its complete utilization can be offered, thereby promoting near-zero waste practices and contributing to a more sustainable future.

1. Introduction

Serenoa repens Small (SR in the following), also known as *Sabal serrulata* or Saw Palmetto, is a low shrubby palm native to North America, where it grows in pine woods and among the sandy dunes in the coastal wetlands of South Carolina, Louisiana, Georgia and Florida (Indena, n. d.; Kwon, 2019).

The first use of the plant extract is attributed to the Native Americans, for genitourinary disturbances (Murray, 1998). Then, in the 1870s, the palm berries started to be investigated for their medicinal properties. Multiple effects were reported, both on the digestive tract and on the reproductive system (ameliorating ovarian dysfunction, decreasing prostate enlargement, etc.). Later, when the synthetic pharmaceuticals started to spread, *Serenoa repens*, like many other phytotherapeutic alternatives, was initially disregarded and only later rediscovered (Geavlete et al., 2011). In the United States, the plant gained recognition as an official remedy in 1906 being first mentioned in the US Pharmacopoeia, followed in 1926 with the inclusion in the US National Formulary (Bennett and Hicklin, 1998). In communication with various commercial entities, the Botanical Adulterants Prevention Program estimates that between 4500 and 8000 metric tons of fresh berries are harvested each year (depending upon fruit set and weather conditions),

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leading to the production of 520 metric tons of extract with a wholesale value of $105 million (Gafner and Baggett, 2017). Various extraction methods are used by different manufacturers, and for this reason each product is not equivalent to another. The liphophilic extracts of its fruits are well known to be effective in the treatment of benign prostatic hyperplasia (BPH) (Bartolomé Ortega et al., 2017; Penugonda and Lindshield, 2013; Priestap et al., 2011; Schantz et al., 2008). To date, the specific mechanism of action is still not completely understood, but it has been proved that the short-chain fatty acids in saw palmetto extracts inhibit the 5α-reductase, a crucial mechanism during benign prostatic hyperplasia treatments (Penugonda and Lindshield, 2013). Nevertheless, a combination of fatty acids, phytosterols, and other bioactive components may be responsible for beneficial effects reported from saw palmetto supplements (Buck, 2004; Penugonda and Lindshield, 2013). Sabalselect®, commercialized both as liquid extract and 30% powder extract, is a supercritical CO_2-derived product of Indena company (https://www.indena.com), nowadays representing a standard reference lipophilic material from SR fruits. Indena report on Sabalselect® product evidences a highly defined chemical profile with reproducible phytochemical characteristics. In particular, the standardized product contains: 85.0–95.0% of fatty acids, 0.01–0.15% of fatty alcohols, 0.25–0.50% of sterols (Indena, n.d.). Besides fatty acids and sterols, which represent the main company's target molecules, literature data on *Serenoa repens* fruits evidence the presence of an interesting class of unsaturated isoprenoid alcohols, named polyprenols, which demonstrated potential biomedical applications, including antioxidant and anti-inflammatory properties (Jommi and Verotta, 1988). Several studies reveal that polyprenols can act as wide-spectrum antiviral agents and positively influence the immune system (N. K. Khidyrova and Shakhidoyatov, 2002a,b; Zhang et al., 2015). Despite the potential biological activities already demonstrated for polyprenols derived from other plant extracts (*i.e. Betula verrucosa, Mallotus japonicus, Aesculus hyppocastanum* (N K Khidyrova and Shakhidoyatov, 2002a,b)) their presence within residual fruits of *Serenoa repens* is not reported and no studies evidenced their potential use and activities concerning BPH treatments. This is likely due to the limited research on specific secondary metabolites isolated from *Serenoa repens*. Currently, most studies on its biological activity for treating benign prostatic hyperplasia (BPH) focus primarily on the full plant extract rather than individual compounds (Tacklind et al., 2009).

In this context, given the high selectivity of the industrial extraction processing of *Serenoa repens* fruits, which employs CO_2 at its supercritical conditions, it is likely that many bioactive compounds of interest are still present in the industrial wastes. The extraction and identification of leftover compounds in the biomass residual, after the industrial extraction, is the object of the present research. Supercritical fluid technologies offer a promising and environmentally sustainable alternative to conventional solvent-based methods for the extraction of non-polar molecules. Notably, the tunable temperature and pressure conditions of different CO_2 densities enable the selective enrichment of targeted species during the extraction process. The technology, employing CO_2 as an extraction fluid, is indeed characterized by low environmental impacts: organic solvents are avoided ensuring safe and selective processes directly on the powder without any pretreatment, with the possibility to recycle the employed CO_2 in the industrial plants (Pereira and Meireles, 2010). Companies are increasingly focusing their attention on supercritical CO_2, first of all for the possibility to avoid any residue of organic solvents in the final product (Karrar et al., 2019). Conventional solvent-based methods, such as hexane extraction, are then avoided together with their well-known safety and toxicological issues (Kimura et al., 1971).

Building upon our previous work on *Cucurbita pepo* L. pharmaceutical residues (Massironi et al., 2022), the objective of this study was to ascertain selective enrichments in obtained extracts starting from the industrial (and already extracted) biomass residues.

To accomplish this, a sequential selective extraction by varying CO_2 densities was carried out, aiming at identified any potential leftover lipophilic compound, targeting specifically first the fatty acids and then the polyprenolic fraction.

The residual biomass was then re-extracted setting the focus on polar compounds, including carbohydrate macromolecules and phenolic compounds. Indeed, only few studies report the effectiveness of SR polar extracts attributable to the presence of phenolic compounds with high industrial interest such as caffeic, cinnamic and ferulic acids, already extensively studied for their antioxidant, anti-inflammatory, and anticancer activities, making them highly valuable in the pharmaceutical sector (Marti et al., 2019; Olennikov et al., 2013; Sapozhnikova et al., 2014). For this step, water at subcritical state was employed, that exhibits altered physical properties by varying its pressure and temperature. Due to its tuned properties, subcritical water extraction (SWE) was demonstrated to be a powerful tool for extracting polar and medium polar compounds such as macromolecules and phenolic compounds from distinct residual biomass (Giombelli et al., 2020; Guthrie et al., 2020; Munir et al., 2018). Compared to other commonly used solid-liquid extraction techniques such as maceration and Soxhlet extraction, SWE has as the advantage to contribute to extraction efficiency and decrease of organic solvents use (Zhang et al., 2020). Furthermore, SWE has demonstrated the ability to preserve the structure of bioactive molecules, such as polysaccharides bounded with phenolic compounds, and therefore their functionality (Rincón et al., 2021; Zhang et al., 2023).

To the best of authors' knowledge, this is the first report combining supercritical CO_2 and SWE to investigate the potential valorization of exhausted *Serenoa repens* residual biomass, representing an opportunity of biorefinery for the pharmaceutical sector. This is a new and practical approach to maximize resource utilization, while avoiding organic solvents, enhancing both the safety and selectivity of the process.

This research brings attention to the presence compounds that remain in the industrial proposing their potential in BPH management (in the case of polyprenols) and bioactive phenolic acids (opening up possibilities in the pharmaceutical and cosmetic industries) connected to their well-documented antioxidant and anti-inflammatory properties.

2. Methods

2.1. Materials

After industrial supercritical CO_2 extraction of commercial oils from *Serenoa repens* fruits, residual biomass (abbreviated in the following as SR) was gently donated by Indena S.p.A.

Carbon dioxide (CO_2) was purchased by Sapio s.r.l (Monza, Italy) with a purity of 99.999%. Trimethylsilyl 2,2,2-trifluoro-N-(trimethylsilyl)acetimidate + chlorotrimethylsilane (99:1) was purchased from MACHEREY-NAGEL (Switzerland). KOH and NaOH, HPLC-grade and analytical grade acetonitrile, water and formic acid were purchased by Sigma-Aldrich Chemicals (Italy). Amberlite IRA 400, HPLC-grade methanol, acetone and acetic acid were purchased by Carlo Erba (Italy).

2.2. Apolar compounds extractions

2.2.1. n-hexane extraction

Dry industrial residuals were ground to fine powder by means of a knife blender (Pulverisette 11, Fritsch, Italy) for 30 s at 10000 rpm. 20 g of SR biomass were added in 250 mL round flask containing 80 mL of *n*-hexane and extracted under magnetic stirring at room temperature. After 6 h the hexane phase was removed by Buchner filtration, and an equal volume of fresh solvent was added to the biomass to continue the extraction procedure under the same conditions. In order to remove mucilage and coarse materials, the suspension was centrifuged at room temperature by using a rotational speed of 6000 rpm for 10 min. The extracts were unified, and the solvent was evaporated by rotary

evaporator (final extract weight of 2.0 g). The sample will be labelled as SR-HEX. Yield was calculated and reported as: % g $_{SR\text{-}HEX}$/g $_{dry\ biomass\ weight}$

2.2.2. n-hexane/acetone extraction

20 g of pulverized residuals were extracted with 250 mL of 1:1 (v/v) hexane:acetone solution under stirring. The same procedure adopted for hexane extraction was repeated. The supernatant was collected and concentrated by rotary evaporator (final extract weight of 0.25 g). The sample will be labelled as SR-HEX/ACETONE. Yield was calculated and reported as: % g $_{SR\text{-}HEX/ACETONE}$/g $_{dry\ biomass\ weight}$

2.2.3. Supercritical CO_2 extraction

Supercritical fluid extractions were performed using a pilot unit SFT110XW supplied by Supercritical Fluid Technologies, Inc. (Newark, DE, USA). It consisted of a 100 cm^3 stainless steel extractor inserted in an oven, a constant pressure piston pump (SFT-Nex10 SCF Pump) with a Peltier Cooler and a collection vessel. 20 g of pulverized residuals were loaded in the vessel for supercritical fluid extractions. Three experimental conditions (referred to as mild, medium and strong) have been applied one by one, with an alternation of static and dynamic cycles, keeping the material in the vessel, to achieve complete extraction of oils components from residuals. Data relevant to each adopted condition have been reported in Table 1. Once the set pressure was reached, a static period was kept for 15 min; then, valves were opened to collect the sample for 30 min in dynamic conditions (CO_2 flow rate = 1.5 mL min^{-1}). The final collected sample will be labelled as SR-scCO_2. A final yield was calculated and reported as: % g $_{SR\text{-}scCO2}$/g $_{dry\ biomass\ weight.}$

2.3. Apolar compounds purification

2.3.1. Separation of neutral and acidic fractions

Neutral and acid components separation was achieved by means of ion-exchange chromatography. Briefly, Amberlite IRA 400 was activated before the use with 150 mL of 1M sodium hydroxide solution, then water was removed flushing methanol. Consequently, 50 mg of oil collected from each extraction method were dissolved in methanol and added to 0.5 g (dry weight) of resin and kept under gentle magnetic stirring for 12 h to achieve the complete acid components adsorption. The mixture was then separated through Buckner filtration from the resin, and the non-adsorbed neutral species were completely recovered by washing the resin with extra methanol. Retained fatty acids were eluted by adding a solution of acetic acid in methanol (1:1). Both acidic and neutral fractions were subjected to rotary evaporation to remove the solvent and recover the samples for weight analysis.

Neutral fractions were then saponified following the Method Cd 3c–91/Method NGD C33-1976. Briefly: KOH ethanolic solution (2M) was added to the neutral fractions. The mixture was heated at boiling point for 2 h under reflux. After the saponification reaction, the obtained unsaponifiable matter was counter extracted in diethyl ether. The obtained mixture was washed 3 times with deionized water to remove the unreacted KOH and the saponified compounds, the solvent was evaporated under vacuum. Extraction yield (%) was gravimetrically quantified in relation to initial biomass dry weight.

2.3.2. Polyprenols isolation

158 mg of unsaponified neutral fraction of SR oil obtained through scCO_2 extraction (SR-scCO_2) was chromatographed on 10% $AgNO_3$/SiO_2 gel (10 g) at medium pressure (flow rate: 4 mL min^{-1}) and eluted with CH_2Cl_2:EtOAc (95:5) and CH_2Cl_2:EtOAc:Acetone (90:5:5). Fraction 1–10 (56.9 mg) and 11–16 (22.6) were separated. Fraction 17–19 (72.5 mg) was repeatedly purified on 10% $AgNO_3$/SiO_2 gel (5 g) using the same eluents and finally purified on SiO_2 column eluting with n-hexane: EtOAc (85:15). Polyprenols enrichments was monitored by TLC and their presence and quantification confirmed through HPLC analysis. Extraction yield (%) was gravimetrically quantified in relation to initial biomass dry weight.

Table 1
Adopted extraction conditions (temperature, pressure and the corresponding CO_2 density).

Run	Oven Temperature (°C)	Pressure (bar)	CO_2 Density (Kg/m^3)<
A (mild)	35	100	700
B (medium)	45	220	830
C (strong)	50	380	913

2.4. Apolar extracts characterization

2.4.1. Gas chromatography-mass spectrometry (GC-MS)

Fatty acids and sterols were analyzed by using an ISQTM QD Single Quadrupole GC-MS (Thermo Fisher, MA, USA) equipped with a VF-5 ms (30 m × 0.25 mm i.d. × 0.25 μm; Agilent Technology, CA, USA). Injection volume: 1 μL, split mode; Oven program: 120 °C for 5 min; then 10 °C min−1 to 200 °C; 5 min holding time; then 20 °C min−1 to 300 °C; 20 min holding time; total run time: 38 min. Helium was used as a gas carrier. Ionization mode: electron impact: −70 eV. Acquisition mode: full scan. To identify the chemical structure of the species eluting, the fragmentation pattern of each peak was compared to https://www.nist.gov/srd/nist-special-database-14 NIST 2014 database.

Before the analysis samples were derivatized by silylation, briefly: 5 mg of dry extract were treated with 0.2 mL of N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) at 60 °C for 1 h. The obtained solution was diluted 1:10 with 0.8 mL of ethyl acetate. NIST 2014 mass spectra library was used to identify isolated sterols and fatty acids in extracts.

2.4.2. High performance liquid chromatography (HPLC)

Polyprenols quantification was performed through HPLC (Waters, Milford, MA, USA) equipped with a Symmetry shield RP18 (100 Å, 5 μm, 4.6 mm × 250 mm) column and a PDA (photodiode array, Waters 2996) detector. The mobile phase was a mixture of acetonitrile +0.1% formic acid (80%) and isopropanol (20%) at a flow rate of 1 mL min^{-1} in isocratic mode (515 HPLC pump, Waters). The injection volume was 5 μL. Total run time for each analysis was 20 min, the wavelength was set at 210 nm. Data were processed with Empower 3 workstations.

Polyprenols identification and quantification was based on comparison with a standard mixture previously isolated from SR fruits oils in our facilities, being polyprenols standards not available in the market. Before samples injections, 5 dilutions of a polyprenols standard methanolic solution (0.2 mg mL^{-1}) were prepared in the range 0.001–0.2 mg mL^{-1}. Standard solutions were filtered (0.2 μm nylon filters) and injected three times in HPLC system. Four main polyprenols were eluted as sharp peaks at retention time of 1.65, 2.15, 2.9 and 4.1 min. In the operative concentration range the trend was linear for each compound, with no saturation effects that could bend the linearity. The area under each peak was quantified by instrumental software and plotted versus the concentration. The best fit of experimental data in the plot "Peak area vs [polyprenol]" was then used for polyprenols quantification in each sample.

2.5. Polar compounds extraction

2.5.1. Subcritical water extraction

Subcritical water extraction (SWE) was performed in an accelerator solvent extractor DIONEX ASE 350 (Thermo Fischer Scientific Inc., USA). Polar compounds extraction from SR residual biomass (after scCO_2) was carried out placing a total of 3 g in 34 mL extraction cell above a layer of diatomaceous earth and sandwiched with cellulose filter. Extractions were performed at 120, 150 and 180 °C (samples have been labelled SR120, SR150 and SR180) for 20 min in a solid:liquid ratio of 1:14. The extracts were submitted to freeze-drying (FreeZone 6,

Labcombo, USA) before further analysis. Extraction yield (%) was gravimetrically quantified in relation to initial biomass dry weight.

2.6. Polar extracts characterization

2.6.1. Molar mass distribution

Determination of molar mass distribution of polar SR extracts was investigated using size-exclusion chromatography coupled with a refractive index detector (SEC-MALLS) in a SECurity 1260 SEC-System (PSS Polymer Standards Service GmbH, GE). Samples were prepared in SEC eluent, consisting of dimethyl sulfoxide with 0.5% LiBr, filtered through 0.2 μm nylon syringe filters and injected into SEC system at a 0.5 mL min^{-1} flow rate at 60 °C, using a column set consisted by a GRAM pre-column (50 mm, 8.00 mm, 10 μm) and analytical GRAM columns with porosity 100-10000 Å (300 mm, 8.0 mm, 10 μm) (PSS Polymer Standards Service GmbH, GE). Pullulan standards (PSS Polymer Standards Service GmbH, GE) with molecular weight with range 342–708.000 Da were used to calibrate the system. Molar mass results were analyzed in terms of Mn (number-average molar mass), Mw (weigh-average molar mass), Mp (molar mass), and polydispersity index (D) values.

2.6.2. Starch content

Starch content was evaluated using Megazyme total starch kit (Wicklow, Ireland). Results were read at 510 nm using glucose as standard.

2.6.3. Carbohydrate fraction analysis

To isolate carbohydrates from SR selected extract, alcohol insoluble residue (AIR) was prepared. Briefly, ethanol (95 % v,v) was added to the dried extracts at 1:10 (extract:ethanol), vigorously stirred and submitted was centrifuged at 4 °C and 4000 rpm for 10 min. The procedure was repeated a total of 5 times. Subsequently, AIR was washed with absolute acetone at the proportion of 1:10 (AIR:acetone), centrifuged, and dried at 40 °C for 24 h.

Afterwards, monosaccharide composition was evaluated in terms of neutral sugars and uronic acids after two-steps methanolysis followed by trifluoracetic acid (TFA) hydrolysis following the method of Wilför et al. (Willför et al., 2009). Determinations were performed in a high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS-6000 system (Thermo Fischer, USA). A column CarboPacTM PA20 (3 × 150 mm) was used for separation at 0.4 mL min^{-1}. The gradient method consisting of Milli-Q water, 200 mM NaOH, and 100 mM NaOH +100 mM NaAc, as eluents A, B, and C, respectively as following: equilibration for 8 min with 1.2% of B; 20 min of 1.2% B; 10.1 min of 50% B; 15.9 min of 100 % C; 3.9 min of 100 % B; 10.1 min of 1.2% B. Quantification was carried out against calibration curve built with neutral sugars (fucose, arabinose, rhamnose, galactose, glucose, xylose, and mannose) along with uronic acids (galacturonic and glucuronic acids).

2.6.4. Glycosidic linkage analysis

Determination of linkage analysis was performed following the method described by Rudjito et al. with modifications (Rudjito et al., 2019). Prepared per-O-methylated methyl alditol acetates were separated and quantified in a GC-MS HP-6890 gas chromatographer (Agilent, CA, USA) and HP-5973 electron-impact mass spectrometer (Agilent, CA, USA), fitted with SP 2380 (Supelco, PA, USA) capillary column. For samples preparation, briefly, dried samples (1 mg) were dissolved in DMSO, flushed with Argon, and kept stirring overnight. Sodium hydroxide freshly grind (200 mg) was added to the sample in excess, flushed with argon and stirred. Methyl iodine (30 μL) was added to the mixture, with repetition of 5 times of 10 min interval, and either three times under stirring, and 2 times under sonication, with argon flushing in between. Resulting methylated carbohydrates were partitioned 3 times with water and dichloromethane, where the organic phase is recovered and dried. Methylated carbohydrates were submitted to two-steps methanolysis (1 M HCl prepared in dried methanol) followed by TFA hydrolysis. After hydrolysis, samples were dried, reduced with sodium borodeuteride ($NaBD_4$) prepared in 1 M ammonia solution at room temperature for 1.5 h, followed by neutralization with 10 % acetic acid prepared in methanol (repeated 3 times) and washed with methanol (3 times). Neutralized samples were submitted to derivatization by acetylation with pyridine and acetic acid anhydride (1:1 v:v, 200 μL) at 100 °C for 60 min. The per-O-methylated methyl alditol acetates were then extracted with ethyl acetate and quantified in comparison with standards, regarding their retention times and fragmentation profile.

2.6.5. Protein content

Protein content in polar extracts was determined according to Bradford et al., using a Bio-Rad protein assay kit (Bradford, 1976). Measurement was carried out at 595 nm against a calibration curve built using albumin (bovine origin, fraction V, 98 % PA) as standard.

Total phenolic compounds (TPC): Determination of total phenolic compounds were performed by their interaction with Folin-Ciocalteu's reagent using an adaptation of the method described by Cicco et al. as follows (Cicco et al., 2009): 25 μL of extracts and 25 μL of Folin-Ciocalteu reagent were added to the microplate wells. After 2 min, 200 μL of sodium carbonate (5%; w:v) was added. Microplates were incubated at 37 °C in the dark for 30 min and read at 760 nm in a FLUOstar Omega (BMG Labtech, Germany) microplate reader. Gallic acid was used as standard, and the results were expressed in mg GAE g^{-1} of dry sample.

Total flavonoids content (TFC): TFC was determined thought the aluminum chloride method according to Shraim et al. with modification (Shraim et al., 2021). Samples (10 mg) were extracted with HCl (2 M) for 30 min at 80 °C and partitioned with ethyl acetate. The extracts were evaporated until dryness, followed by resuspension in methanol. The assay was performed in microplate method as follow: 80 μL of methanol, 25 μL of each sample or standard solution and 6 μL of $NaNO_2$ (1.0 mol L^{-1}), were added to each well. After 3 min, 6 μL of $AlCl_3$ (100 g L^{-1}) was added to the mixture. After 3 min, 40 μL of NaOH (1.0 mol L^{-1}) is added and the final volume adjusted to 200 μL with methanol. The microplate was stored in the dark for 40 min at room temperature and read at 485 nm in a microplate reader FLUOstar Omega (BMG Labtech, Germany).

HPLC: Phenolic compounds were determined by high-performance liquid chromatography (HPLC) using a Waters HPLC system constituted by a separation module (Waters 2695, USA) coupled to a photodiode array detector (Waters, 1996; USA). A ZORBAX StableBond C18 column (Agilent Technologies, USA) was used for separation. A gradient method was performed with acetonitrile 1% acetic acid (v/v) as eluents A and B, respectively. The elution gradient was used as follow: 0–12 min (5% B), 12–23 min (10 % B), 23–25% (50 % B), 26–28 (65 % B), 28–45 min (5 % B) in a solvent flow of 1.0 mL min^{-1} and injection volume of 25 μL. Calibration was performed using hydroxybenzoic acids (gallic, gentisic, and vanillic acids), hydroxycinnamic acids (caffeic, ferulic, sinapic, p-coumaric and cinnamic acids) and flavonoids (catechin, rutin, myricetin, luteolin, quercetin, and kaempferol). All compounds were commercial standards provided by Merck Chemical (Sweden). Individual phenolic acids and flavonoids were quantified based on the retention times of the standards at 325 nm, apart from catechin and kaempferol which was determined at 375 nm. Prior to analysis, SR extracts were submitted to saponification with 2M sodium hydroxide at 30 °C overnight, followed by ethyl acetate partition at proportion of 1:2 (sample: ethyl acetate) repeated three times, were used.

2.6.6. In vitro antioxidant activity

The antioxidant activity of polar SR extracts was evaluated based on the radical scavenging activity of the radical ABTS (2′2-azinobis (3-ethyl-benzothiazoline-6-sulphonate) and radical DPPH (1,1-diphenyl-2-picrylhydrazyl). The results were expressed in Trolox equivalent (mg_{TE}

g_{dw}^{-1}), which were calculated taking into account samples concentration able to scavenge 50% of studied radicals. All analysis were performed in triplicates.

ABTS radical scavenging activity of extracts was estimated according to the method described by Re et al. with some adaptations (Re et al., 1999). Samples and standards concentrations were adjusted to have less than 80% of the absorbance of the control. The reaction was carried out by adding to 20 μL of the sample, 200 μL of radical ABTS 7 mM, previously prepared in 2.45 mM of potassium persulfate and diluted with ethanol. Microplates were incubated in the dark at 30 °C for 25 min, and read in the microplate reader FLUOstar Omega (BMG Labtech, Germany) with absorbance at 734 nm.

Evaluation of scavenging activity against the radical DPPH was carried out according to the method proposed by Brand-Williams, Cuvelier & Berset with few adaptations to the micro assay (Brand-Williams et al., 1995). Briefly, 100 μL of samples or standards are mixed with 100 μL of the radical DPPH 0.2 mM and reacted in the dark for 30 min. Microplates were read in FLUOstar Omega (BMG Labtech, Germany) with the absorbance at 517 nm at the time 0 and 30 min.

2.6.7. Antimicrobial activity

Antimicrobial activity of SR polar extracts was investigated against strains of typical pathogens including a Gram-negative *Escherichia coli* (CCUG 10979) and two Gram-positive *Listeria innocua* (CCUG 15529) and *Bacillus cereus* (CCUG 7414) representatives. For this, extracts were resuspended in DMSO 50% (v:v) in the range of 100-6.25 mg mL^{-1}. *E. coli* and *L. innocua* strains were cultivated in tryptic soy broth (TSB), while *B. cereus* was cultivated in Lysogenic broth (LB) at 37 °C for 24 h. Optical density at 600 nm (OD_{600}) of microorganism media was adjusted to McFarland 0.5 standard and diluted to approximately 10^5 colony forming units [CFU] mL^{-1}. Bacterial media was used to inoculate different concentrations of extracts and then incubated for 24 h at 37 °C. The minimum inhibitory concentration (MIC), which is the lowest concentration of extract that can inhibit bacterial growth, was visually determined. Cultures with no visual bacterial growth were inoculated on the surface of solidified Mueller Hinton agar plates for verification of viable cells. The number of colonies was counted after 24 h of incubation at 37 °C. Samples with ability to kill bacteria strains were those in which <10 CFU was formed.

2.6.8. Cytotoxicity assessment

The cytotoxicity of selected *SR* extracts were investigated in human intestinal epithelial cells (Caco-2) and human keratinocytes (HaCaT) cell lines through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, ThermoFischer, Sweden) assay. The MTT working solution was prepared at 5.5 mg mL^{-1} in phosphate-buffered saline (PBS; Sigma-Aldrich).

2.6.9. Cell culture

Caco-2 cells and HaCaT cells, obtained from cell Lines Service (Eppelheim, Germany), were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Scientific, Sweden) media, supplemented with 10 % (v/v) fetal calf serum (FCS; Sigma-Aldrich), 1 % glutamine, and 1% of penicillin/streptomycin (P/S). The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Culture media was changed every 2–3 days, and the cells were propagated weekly after treatment with Accutase (Gibco Scientific, Sweden) solution.

2.6.10. Cell viability

The biocompatibility of SR polar extracts upon Caco-2 and HaCaT cell lines were evaluated using MTT colorimetric assay. Briefly, Caco-2 and HaCaT cells at density of $1x10^5$ and $3x10^4$ cell mL^{-1}, respectively, were seeded in the wells of 48-well plates and allowed to adhere. Subsequently, culture media was removed and the cells were treated with different concentrations of the extracts (0.5–2.5 mg mL^{-1}), previously diluted in PBS. After 24 h, MTT working solution was added to each well to reach the final concentration of 0.5 mg mL^{-1}, and the plates were incubated for 4 h, followed by the complete removal of media and solubilization of formazan salt with 1 mL of dimethyl sulfoxide (DMSO). Triton-x 2% (v/v) was used as control positive for cytotoxicity, while cells untreated were used as control negative, and media was used as blank. Cell viability was determined through the absorbance assessment at 595 nm microplate reader FLUOstar Omega (BMG Labtech, Germany).

3. Results and discussion

3.1. Extraction results: apolar extracts

The starting biomass was already extracted by the company by means of supercritical CO_2 (extraction parameters are confidential) to obtain the active principle of the pharmaceutical oil. Aiming at verifying the effectiveness of the industrial extraction to exhaust the oil content from the biomass, for explorative and optimization purposes, three experimental conditions, referred to as mild (35 °C and 100 bar), medium (45 °C and 220 bar) and strong (50 °C and 380 bar), have been sequentially applied.

Two cycles (two alternations of static and dynamic cycles) were performed to reach a plateau of extraction yield in mild conditions (35 °C and 100 bar, blue points in Fig. 1), two cycles were performed to reach the plateau of extraction yield in medium conditions (45 °C and 220 bar, green points in Fig. 1) and finally five cycles were performed to reach the plateau of extraction yield in strong conditions (50 °C and 380 bar, red points in Fig. 1).

The final collected sample, labelled as SR-scCO_2 was compared to the extract obtained by conventional solvent-based method, employing *n*-hexane (labelled as SR_HEX).

Mild and medium conditions in supercritical CO_2 (blue and green points in Fig. 1) did not allow to obtain considerable yields in comparison to the applied strongest condition (red line in Fig. 1). The total extraction yield was 1.2% (sample labelled as SR-scCO_2, 0.28 g). Indeed, among the three sequential scCO_2 extracts, only the last one (T = 50 °C, p = 380 bar) was in sufficient amount to be considered for the further separation through ion exchange resin into neutral and acidic fractions. It resulted composed of an almost equal percentage of free fatty acids and neutral compounds (55% and 45% respectively, see Table 2).

The *n*-hexane extraction yield (*SR_HEX*, 0.4%), performed for comparative purposes with a conventional and environmentally

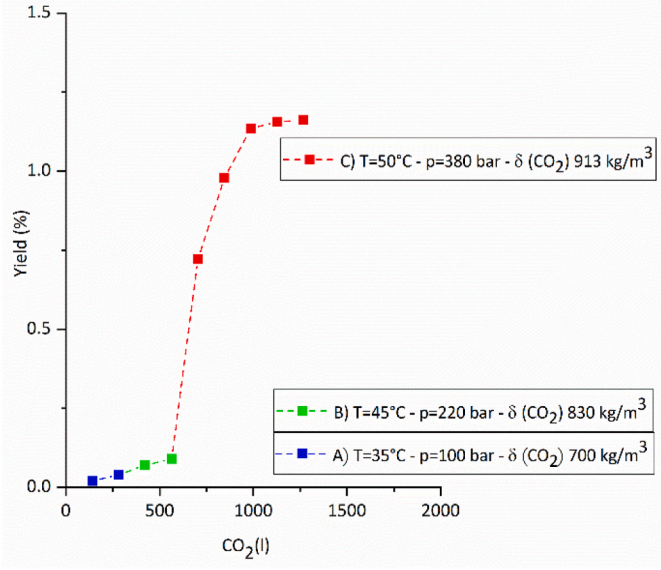

Fig. 1. Extraction kinetics in sequential scCO_2 extractions from SR biomass.

Table 2
Main composition of extracts obtained from *Serenoa repens* fruits residuals (dw:dry weight).

Biomass	Extraction conditions	Extraction Yield %	% Free fatty acids	% Neutral fraction	% Unsaponifiable matter
SR Residuals	A) T = 35 °C p = 100 bar d_{CO2} 700 kg/m^3	/			
	B) T = 45 °C p = 220 bar d_{CO2} 830 kg/m^3	/			
	C) T = 50 °C p = 380 bar d_{CO2} 913 kg/m^3	1.2 ± 0.2 (0.28 g)	55 ± 3 (% extract dw) 0.77 ± 0.04 (% biomass dw)	45 ± 4 (% extract dw) 0.63 ± 0.06 (% biomass dw)	2.9 ± 0.5 (% extract dw) 0.040 ± 0.007 (% biomass dw)
	n-hexane	0.4 ± 0.1% (0.08 g)	74 ± 8 (% extract dw) 0.27 ± 0.03 (% biomass dw)	26 ± 4 (% extract dw) 0.10 ± 0.02(% biomass dw)	1.4 ± 0.3 (% extract dw) 0.006 ± 0.001(% biomass dw)

unsustainable solvent-based method (Li et al., 2006), resulted significantly lower with respect to SR-scCO$_2$ extracts (1.2%) and, for this reason, discarded in the subsequent extraction cascade. In this case, matrix effects due to the lower mass transfer kinetics in solvent-based conditions, could have badly affected the extraction yield. The easy diffusivity of the supercritical fluid inside the porosity of the SR biomass could have had beneficial effects to enhance the yield. The lower content of the unsaponifiable fraction in the *n*-hexane extract (Table 2, 1.4%, compared to 2.9% of the scCO$_2$ extracts) could be explained by the presence, in this fraction, of sterols and, mostly, isoprenoid alcohols, as assessed by the literature (Iehlé et al., 1995). These species, characterized by a higher polarity, cannot be easily extracted by an apolar solvent such as *n*-hexane. In supercritical conditions, the supercritical fluid density (that is related to the specific couple of chosen parameters, namely temperature and pressure) slightly but effectively affects the polarity of the fluid hence shifting the extractability window. This process is also facilitated by the so-called "dragging out phenomenon" during the dynamic stage (Champeau et al., 2020; Marzorati et al., 2023), when CO$_2$ is flushing out from the vessel, pulling less expected compounds out from the vessel. In addition, the presence of readily soluble species (such as fatty acids and glycerides) could act as co-solvents, again assisting co-extraction of more polar species.

3.2. Serenoa repens acid and neutral fraction compositions

The GC-MS analysis of the isolated acid components from SR-scCO$_2$ and SR_HEX confirm the presence of short-chain fatty acids with similar relative abundances with respect to commercial and literature SR oils. Relative fatty acids abundances are reported in Table 3 with a standard deviation of triplicate analysis of all obtained extracts.

The adopted extraction method seems not to interfere with fatty acids' relative abundances; only small changes are observed among obtained extracts.

Derivatized sterols of extracts from SR fruits residuals were identified by peaks comparison with the NIST2014 mass spectra library via detection of the parent molecular ions $[M]^+$, $[M-15]^+$ (loss of the methyl terminal group), $[M-90]^+$ (the loss of the $(CH_3)_3SiOH$ group) and $[M-105]^+$ (loss methyl group and $(CH_3)_3$ SiOH). The relative abundances of revealed species were calculated and reported in Table 4, where it is displayed that β-sitosterol constitutes more than 50% of total sterols content. Although SR-scCO$_2$ and SR-HEX samples displayed almost identical sterols composition, SR-scCO$_2$ presents higher contents of unsaponifiable matter (3 % of total extract weight) than SR-HEX. Nevertheless, several β-sitosterols and more in general Δ5-sterols sources are present in nature and the process of their extraction and isolation is inexpensive and well-known (Ye et al., 2010).

Table 3
Fatty acids' relative abundances of SR extracts.

Fatty acids	Relative abundances %		
	SR-scCO$_2$ (*d_{CO2}* 913 kg/m^3)	SR_HEX	*SR reported literature.* (Indena, n.d.; Penugonda and Lindshield, 2013; Priestap et al., 2011)
Capric acid (C10:0)	2.3 ± 0.1	3.1 ± 0.5	1–3
Lauric acid (C12:0)	28 ± 2	25 ± 0.8	20–30
Myristic acid (C14:0)	5.1 ± 0.3	4.3 ± 0.2	3–6
Palmitic acid (C16:0)	13 ± 1	13 ± 2	10–15
Stearic acid (C18:0)	0.7 ± 0.2	<1	0.1–1
Oleic acid (C18:1)	38 ± 3	39 ± 4	25–45
Linoleic acid (C18:2)	14 ± 2	15 ± 1	10–20

3.3. Polyprenols from Serenoa repens residuals

Besides sterols, polyprenols were expected to be present in SR unsaponifiable matter fraction. The composition of the unsaponifiable matter of SR-scCO$_2$ oil has been determined after the isolation of its main constituents by means of $AgNO_3/SiO_2$ chromatography exploiting the silver activity to act as π acceptor allow to separate molecules that do not contain a π donor (or fewer π donors) eluting them faster from molecules which contain double bonds such as polyprenols (Ndunda et al., 2015). Four main fractions have been isolated 1–10 (84 mg), 11–16 (22.6 mg), 17–19 (72.5 mg) and 20–30 (6 mg). Fractions 1–10 and 11–16 were analyzed by means of GC-MS: 1–10 resulted in a heterogeneous mixture of residual fatty acid after the saponification reaction and sterols; 11–16 was mainly constituted by triterpenoids alcohol and sterols whose relative abundances and structures have been listed in Table 4. Finally, fraction 89–95 has been further purified by means of SiO_2 column yielding a sole polyprenols mixture (8.4 mg) whose composition has been investigated by HPLC analysis allowing to identify the presence of four main polyprenols, whose structure is reported in Fig. 2. Fig. 3 reports the HPLC chromatogram. Polyprenols identification and quantification was based on comparison with a standard mixture previously isolated from SR fruits oils in our facilities.

Polyprenols from plant biomass have different cis/trans configurations and different number of isoprene units, both strongly affecting the retention time and allowing separation of the mixture in HPLC analysis. Such isoprenic unit sequence is recurrent in the Angiospermae plants' group to which *Serenoa repens* belongs (K. Ibata et al., 1984a; Ibata et al., 1984b; Jommi and Verotta, 1988).

Four main peaks were detected from HPLC analysis at 210 nm corresponding to: a) C30, 5 isoprene units; b) C35, 6 isoprene units; c) C40, 7 isoprene units; d) C45, 8 isoprene units, where C corresponds to the number of carbons in polyprenols chain.

(Figs. 2 and 3). Peaks attribution was based on precedent work from Jommi and co-workers who isolated the four polyprenols from *Serenoa repens* and elucidated their structures and number of isoprene units by NMR and GC-MS analysis (Jommi and Verotta, 1988).

The analysis of chromatograms obtained through HPLC analysis evidenced the presence of four main peaks at the retention time of polyprenols standard mixture.

In terms of quantification, SR-scCO$_2$ extract displayed the highest percentage of polyprenols. In the *n*-hexane and in *n*-hexane/acetone extract (this one reported as the optimal extraction solvent for polyprenols) only traces of polyprenols were detected (Alaydi et al., 2021a;

Table 4
Fragmentation ions used for the identification of trimethylsilyl ether sterols.

Sterols	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-105]^+$	Sterol relative abundance (%)	SR oils from literature (Heim et al., 2018; Marti et al., 2019) Sterol relative abundance (%)
Cicloartenol	498	483	408	393	13 ± 1	10–15
Campesterol	483	468	393	378	12 ± 1	10–14
Stigmasterol	484	469	394	379	3.8 ± 0.1	2–5
β-sitosterol	486	471	396	381	56 ± 2	40–60
24-methylenecycloartanol	498	483	408	393	8.7 ± 0.3	6–10
Lupeol	498	483	408	393	7 ± 1	5–8

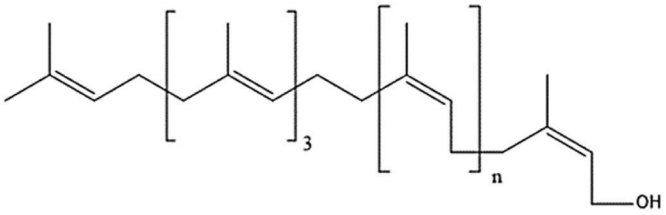

Fig. 2. Polyprenols structure in *Serenoa repens* fruits oils.

Jommi and Verotta, 1988) (Table 5).

These data evidence polyprenols high-affinity to $scCO_2$ at adopted extraction conditions, resulting in composing more than 55% of the total unsaponifiable

matter of SR-$scCO_2$ extracts. Polyprenols were demonstrated to positively affect several adverse conditions such as high cholesterol levels in blood, diabetes, gout, etc. Their potential exploitation as bioactive compounds has never been carefully evaluated due to the extraction and purification costs connected to their low plant concentration (Alaydi et al., 2021b).

Polyprenols extraction from SR residuals by means of supercritical technologies could represent a new alternative and more efficient procedure for polyprenols extraction and isolation. To the best of our knowledge, solely Jommy and co-workers, in 1988, reported the total polyprenols content obtained from the hexane extract of Serenoa repens with extraction yield of 0.0035% of total dry weight and 0.38% of total oil content (Jommi and Verotta, 1988).

3.4. Extraction results: polar extracts

The feasibility of subcritical water extraction as green method for valorization of SR biomass after recovering of lipids and polyprenols through supercritical CO_2 was assessed. A proportional increase in the extraction yield was observed with the rise in processing temperature from 120 °C to 180 °C (Fig. 4). This trend might be correlated with the subcritical water hydrolytic power that tends to increase with processing parameters, such as temperature (Cocero et al., 2018; Zhang et al., 2020). In this way, the presence of soluble matter increases with the hydrolytic power, which in turn affects the extraction yield.

Molar mass distribution for SR extracts shows a unimodal distribution with a high molecular mass population centered between 10^3-10^4 Da. Furthermore, small variations on number-average molar mass (Mn) of SR extracts were observed with an increase in temperature. Conversely, it was accompanied by a notable increase in the weigh-average molar mass (Mw), particularly for SR150 extracts (Table 6). The result indicates that the increase in temperature influences the polymeric chain profile, resulting in SR molecules with a slightly higher polymeric chain at 150 °C compared to 120 °C, followed by a decrease at 180 °C. This outcome is expected, as higher temperatures facilitate the easier recovery of oligomers and monomers [47] and peptides and amino acids [48] as autohydrolysis result.

The carbohydrate content of the SR extractions by SWE range of 136.05–253.39 mg g^{-1}, as displayed in Table 6. The carbohydrate profile of SR extracts changed in accordance with processing temperature, decreasing progressively the presence of glucose content, attributed mainly to the presence of starch, to give place to mannose as the main monosaccharide from 120 °C up to 180 °C. This profile is different from the observations found by Wagner & Flachsbarth (Melca Rareseah et al., 1981), that reported for isolated polysaccharides from aqueous SR extracts the presence of galactose as the main monosaccharide followed by arabinose, with minor presence of mannose. Ibrahim et al. (2017) reported that in ethanolic extracts of SR berries, galactose is the main monosaccharide followed by mannose in a proportion of 3:1 (galactose: mannose). The results observed here suggested that mannooligosaccharides and/or mannans might be extracted easily at moderated apolar conditions as observed in SWE at high temperature and in ethanolic extracts. In addition, the proportion of galactose:

Table 5
Polyprenols quantification results.

Extract	Polyprenols oil content (% extract dw)	Polyprenols yield (% biomass dw)
SR-HEX	<0.1	<0.001
SR-HEX/ ACETONE	0.3	0.005
SR-$scCO_2$	1.6	0.025

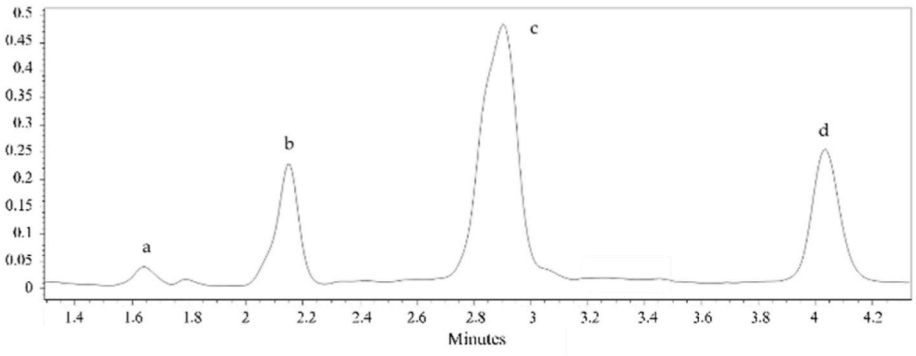

Fig. 3. HPLC chromatogram at 210 nm of standard polyprenols mixture isolated from *Serenoa repens* fruits extract.

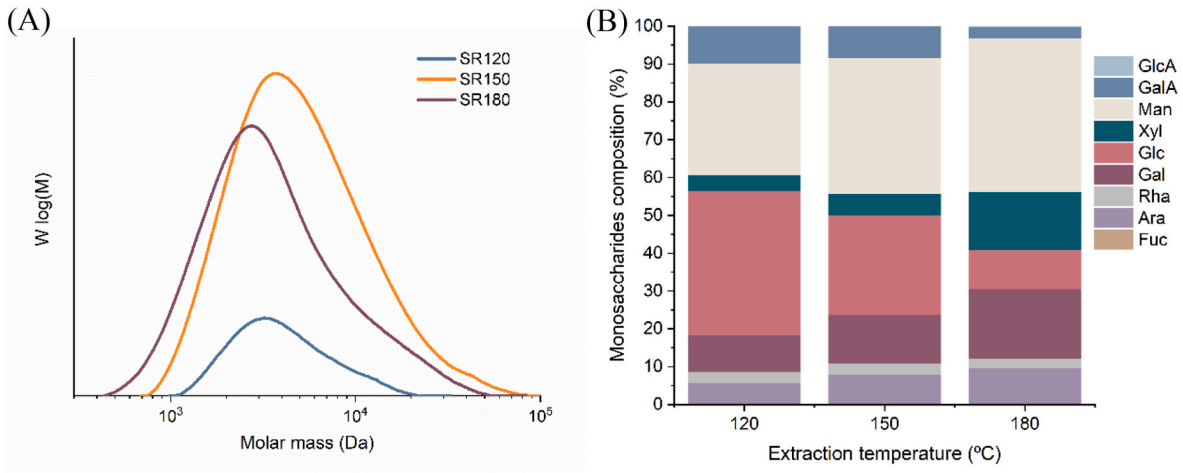

Fig. 4. Molecular distribution (A) and relative monosaccharides composition (B) of polar extracts from *Serenoa repens* obtained by SWE under different conditions.

Table 6
Chemical composition of *Serenoa repens* polar extracts obtained by SWE under different conditions.

Chemical composition	Extraction temperature		
	120 °C	150 °C	180 °C
Yield (%)	16.02 ± 2.94	22.26 ± 0.93	26.63 ± 2.88
Mn (kDa)	3.44	3.83	2.79
Mw (kDa)	4.73	7.07	5.35
Mp (kDa)	3.21	3.63	2.76
Polydispersity index (D)	1.37	1.85	1.92
Carbohydrate content (mg g^{-1}_{dw})	136.05 ± 13.33	173.02 ± 36.13	253.39 ± 54.32
Starch (mg g^{-1}_{dw})	41.18 ± 1.29	40.58 ± 3.14	34.43 ± 4.38
Soluble proteins (mg g^{-1}_{dw})	22.29 ± 3.67	182.73 ± 6.58	125.84 ± 28.21
Phenolic compounds			
Total phenolic compounds (mg g^{-1}_{dw})	127.63 ± 9.64	235.66 ± 5.56	347.80 ± 70.95
Total flavonoids content (mg g^{-1}_{dw})	16.94 ± 4.62	34.70 ± 4.46	22.11 ± 4.03
HPLC (mg g^{-1}_{dw})	0.80 ± 0.10	1.77 ± 0.01	2.10 ± 0.10
Antioxidant activity			
ABTS (mg_{TE} g^{-1}_{dw})	75.80 ± 1.69	144.14 ± 26.06	39.35 ± 9.07
DPPH (mg_{TE} g^{-1}_{dw})	0.031 ± 0.006	0.088 ± 0.004	0.036 ± 0.004

mannose of 1:2 might indicate the presence of galactomannans (GMs) or galactoglucomannans (GGMs) as the main polysaccharide in SR polar extracts, as suggested previously in the literature (Monteiro et al., 2019; Moreira and Filho, 2008).

Regarding the protein content of SR polar extracts, Table 6 indicates that SR exhausted biomass remains a viable source of proteins, and more probably peptides and free amino acids at high temperatures. A prominent increase of protein content is achieved by elevating the extraction temperature from 120 °C to 150 °C, resulting in extracts with eight times the initial concentration. Wagner & Flachsbarth (Monteiro et al., 2019) reported a protein content in SR fractionated extracts of 30 mg g^{-1}, a value that is close to the observed in the present study for extracts obtained by SWE at 120 °C. Nonetheless, 180 °C seems a critical temperature, since the protein content tends to decrease, possibly because of denaturation in function of the high temperature (Awaluddin et al., 2016) or even due to the hydrolysis of the polymeric chains (Barea et al., 2023).

Apart from macromolecules, SR is also a source of phenolic compounds (Olennikov et al., 2013). This aspect is also interesting for biorefinery approaches, given high added value products attributed to the antioxidant activity of the biomass. In the present study, temperature played an important role in phenolic compounds extraction for SR polar extracts (Table 6). Progressive increase of temperature favored the extraction of phenolic compounds from SR biomass. The decrease of water viscosity and polarity contributes improve water diffusivity and decreasing the adhesive forces among bioactive molecules and the cell wall matrix (Cocero et al., 2018; Gonçalves Rodrigues et al., 2019). Previous results for SR demonstrated values of TPC among 6.35–19.34 mg_{GAE} g^{-1} and TFC ranging from 0.33 to 0.98 mg_{RE} g^{-1} or 1.10 mg_{CE} g^{-1} (Barakat et al., 2020a; Olennikov et al., 2013). The discrepancy in the results might be related to the extraction technique. SWE has demonstrated best performances for phenolic compounds extraction in distinct biomasses when compared with traditional extraction techniques (Luo et al., 2018; Mesquita et al., 2022; Rambabu et al., 2022).

A close look at the phenolic compounds profile (Fig. 5) evaluated by HPLC reveals that SR polar extracts are majorly composed of the hydroxybenzoic acids gentisic and vanillic acid, and by catechin, rutin, and kaempferol as the main representatives of flavonoid compounds. This find agrees with Barakat et al. (2020a) that reported hydroxybenzoic acids as the major phenolic acids in SR extracts. Besides, the authors also detected the presence of catechin, rutin and kaempferol. Compared to phenolic compounds determined by Folin-Ciocalteu, HPLC results were lower. This discrepancy might be explained not only by the non-specificity of the Folin-Ciocalteu assay as pointed previously (Sánchez-Rangel et al., 2013) but also due to the lack of standards for determination of phenolics in the very complex structure of polar extracts from *Serenoa repens*. Regardless of the difference of values between the two techniques, the phenolic compounds in SR polar extracts determined by HPLC followed a similar trend than total phenolics compounds quantified by Folin-Ciocalteu, increasing in function of temperature. This is expected, since at SWE processing, hydrolytic reactions lead to release of bonded phenolic acids linked to low molecular or cell wall structural components. Particularly, 170 °C is a temperature high enough to cleave phenolic-polysaccharides ether bonds, increasing the diffusion of phenolic acids during extraction (Chiremba et al., 2012; Tuyet Lam et al., 1992), which explain the high content of phenolic compounds obtained for SR extracts at 180 °C.

3.4.1. *In vitro antioxidant activity*

Antioxidant activity of SR polar extracts was assessed in terms of ABTS and DPPH radical scavenging (Table 6). A high antioxidant activity was observed for SR polar extracts when compared with previous reports for SR extracts prepared using relatively apolar solvents, such as acetone and ethyl acetate (Barakat et al., 2020a; Ibrahim et al., 2017). Moreover, SR extracts demonstrated a good performance in terms of antioxidant capacity in comparison with commercial additives such as gallic acid (Fig. SI1). The scavenging activity of SR extracts against the

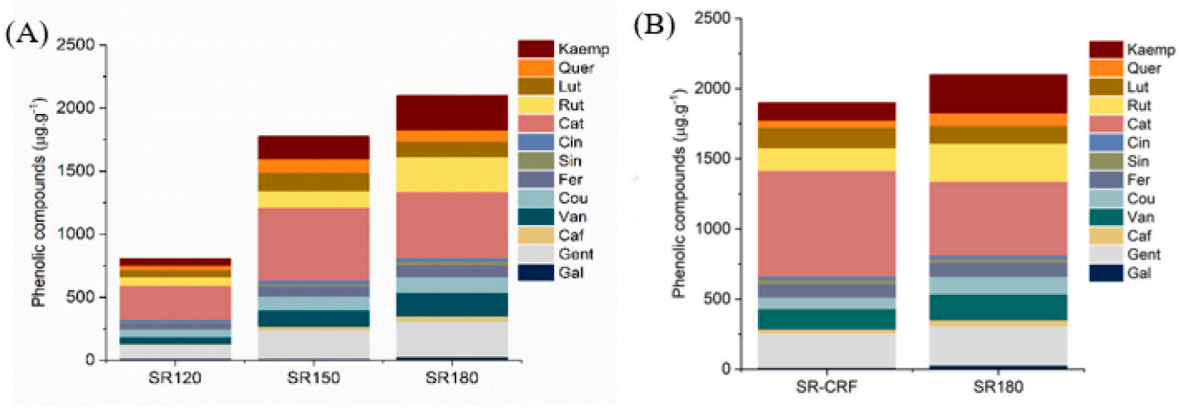

Fig. 5. (A) Phenolic compounds profile of *Serenoa repens* polar extracts obtained by SWE under different conditions and (B) comparison between the phenolics present in the carbohydrate-rich fraction recovered from SR180.

free radical ABTS followed the same trend as observed for the DPPH free radical, indicating that the highest antioxidant activity was achieved at 150 °C as optimal processing temperature. Barakat et al. (2020a) previously demonstrated a strong correlation between phenolic compounds and the antioxidant activity of SR extracts. However, this trend was not observed for SR extracts obtained at 180 °C, suggesting a potential initial degradation of thermally labile bioactive compounds at this temperature, which aligns with the results observed for total flavonoid content, and corroborated with those observed by HPLC, in which the decrease of catechin and quercetin was determined.

3.4.2. Antimicrobial activity

Regarding the antimicrobial property of SR polar extracts, all bacterial strains tested exhibited susceptibility to extracts. The minimum bactericidal concentration (MBC) for 180 °C extracts was 25 mg mL^{-1} against *E. coli*, 100 mg mL^{-1} against the Gram-positive, *B. cereus* and 12.5 mg mL^{-1} against *L. innocua*. While, for extracts obtained at 120 and 150 °C, no bactericidal activity was observed against the Gram-negative *E. coli*. However, those extracts presented a similar behavior against *L. innocua* and *B. cereus*, with MBC of 100 and 15 mg mL^{-1}. When results were compared with other natural compounds like allicin, derived from garlic, the concentration used against *E. coli* and *B. cereus* are lower, around 0.5–1 mg mL^{-1} (Feldberg et al., 1988). However, in this case the compound was not extracted in a sustainable way and was not highly purified in contrary with the fractions obtained in this study. Notably, extracts obtained at 180 °C demonstrated the highest efficacy against both Gram-positive and Gram-negative strains. The results suggest that the temperature increases during the processing of SR extracts enhances the retrieval of antimicrobial compounds with increased effectiveness capable of penetrating the out membrane of Gram-negative bacteria, known by its resistance because of the richness of lipopolysaccharides, contrary to the Gram-positive. The efficacy of SR180 extracts may be attributed to the elevated concentration of phenolic compounds, potentially augmented by the synergistic interaction among them (Leyva-Jimenez et al., 2019). Another explanation could be related to the presence of Maillard reaction products, reported as antimicrobial compounds previously in the literature (Feng et al., 2021). Antibacterial property against Gram-positive and Gram-negative strains were previously reported for apolar extracts (Abdel-Aty et al., 2023; Barakat et al., 2020b).

3.4.3. Investigation on SR180 fraction

From SR polar extracts, SR180 stood out as the optimal condition based on its performance in terms of bioactivity and carbohydrate content. This extract was then employed for carbohydrates valorization and characterized in terms of their potential bioactivity. The carbohydrate-rich fraction (SR-CRF) was characterized in terms of molecular weight, carbohydrate profile according to the monosaccharide composition and structural linkage analysis, along with the potential cytotoxicity (Table SI1 and Fig. 6).

A relevant high yield was obtained for SR-CRF (82.56 %, Table SI1), demonstrating the feasibility of carbohydrates recovery from SR180 extract. Apart from the yield, the molar mass of the recovered fraction was 3.17 kDa, accompanied by an increase of the polydispersity index from 1.92 to 2.25, despite a unimodal distribution (Fig. SI2). The results indicate an increase of heterogeneity due to the presence of molecules with different molar mass sizes inside the same population. Wagner & Flachsbarth (Melca Rareseah et al., 1981) isolated acidic carbohydrates from SR polar extracts with a molar mass of 10^5 Da, which is lower than the observed here for SR-CRF, demonstrating the feasibility of SWE process to recover molecules with high polymeric degree in relation to moderated water percolation conditions.

To elucidate the glycosidic pattern of the SR-CRF recovered from SR180 extract a linkage analysis was performed. The major composition was revealed as t-Manp, t-Glcp, 4-Manp, 4-Glcp, 4,6-Manp, 2-Galp, 4-Galp and t-Galp, which confirms the presence of GGM-oligosaccharides, agreeing with the monosaccharide composition that revealed a proportion of 73.03 % of the total carbohydrate composition. The identified GGM was structurally characterized by a ratio of Man:Glc:Gal of 3:1:2. The linkage analysis showed that aside from GGM, the presence of other oligosaccharide being constituted by xylanosyl (21.10%) and arabinosyl (12.50%) moieties, including 4-Xylp, 2,4-Xylp, 3,4-Xylp, 2,3,4-Xylp and t-Xylp, linkages, which indicates a xylan backbone. While the presence of 5-Araf, 3,5-Araf, 2,5-Araf and t-Araf linkages and the moieties proportion suggest branch-point residues formed by arabinosyl units. The combination of xylan backbone and arabinofuranosyl ramifications confirm the presence of arabinoxylan (AX), representing 26.23% of the total carbohydrate composition. The result is consistent with the monocot primary cell wall nature of SR plant (Pitman, 1993). At the same time, the coextraction of AXs along with GGM was also previously reported on hot water extracted spruce sawdust (Halahlah et al., 2023).

The phenolic fraction remaining during the recovering of the SR-CRF from SR180 extracts was complementarily evaluated (Table SI1 and Fig. 6B). A high portion of phenolic compounds remains retained along with the carbohydrate fraction, represented by 1.75 mg g^{-1} in comparison to 2.0 mg g^{-1} of phenolic compounds from the crude extract. Besides, an increase on the proportion of catechin and luteolin, after recover of carbohydrates, indicates that these flavonoids are important phenolic compounds that might remained attached to the GGM- and/or AXs-oligosaccharides structure. It is important to highlight that the conjugation of phenolic compounds to the carbohydrates might confer certain bioactivity to these molecules (Halahlah et al., 2023). Nevertheless, the decrease of total phenolic compounds observed in

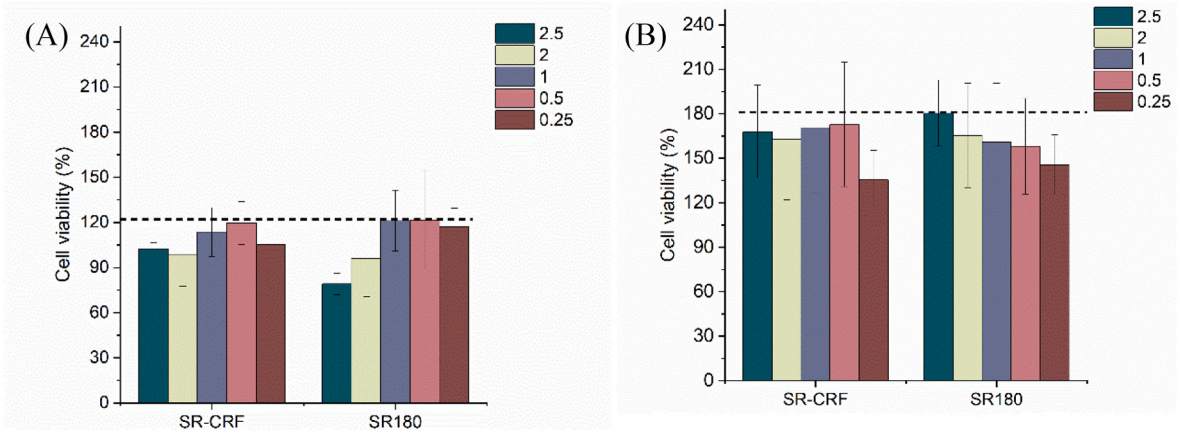

Fig. 6. Cell viability assay of carbohydrate-rich fraction recovered from SR180 extract against Caco-2 (A) and HaCaT (B) cell models.

comparison to the crude extract might be explained to phenolic molecules in the free form or easily leached out during the ethanol/acetone precipitation of the SR180 carbohydrates.

To access possible applications of the carbohydrates recovered from SR on cosmetics or food industry, their cytotoxicity was investigated *in vitro*. For this, human keratinocytes (HaCaT) and colon epithelial (Caco-2) cell lines were selected as the main cell models representatives (Fig. 6A and B).

Concerning the Caco-2 cytocompatibility (Fig. 6A), the SR-CRF shown absence of cytotoxicity from 2.5 to 2.0 mg mL^{-1}. In concentrations from 1.0 to 0.5 mg mL^{-1}, an increase in cell growth was observed, which might be a function of the stimulus provided by the carbon source. In contrast, with SR180 crude extract, a prominent decrease in the cell proliferation is observed at the highest concentration (2.5 mg mL^{-1}), indicating a level of cytotoxicity around 20 %. While at lower concentrations from 1.0 to 0.5 mg mL^{-1}, the same trend of SR-CRF, on the stimulation of cell proliferation was observed for the crude extract, detected in terms of cell viability. Likewise, SR-C presented no inhibitory activity against HaCaT cell lines (Fig. 6B), with the proliferation of cells around 161.86 ± 15.25% in comparison to 162.02 ± 12% of cell viability for the SR180 crude extract. Moreover, it is important to note that despite the non-cytotoxic effect on both cell lines, the cell proliferation was higher to HaCaT than to Caco-2 cell lines, suggesting an interesting potential for wound healing promotion. Additionally, the prebiotic properties of mannan oligosaccharides already demonstrated previously (Ghosh et al., 2015) and the non-cytotoxicity property, highlights the potential of this functional byproduct from *SR* for applications in food additives and supplements.

4. Conclusions

The study integrates both supercritical CO_2 extraction and subcritical water extraction in a cascade process, investigating a highly efficient and environmentally sustainable approach to biomass valorization. Initially, sc-CO_2 extraction was employed to selectively recover lipophilic compounds such as lipids and polyprenols, which are both valuable for their health benefits and industrial applications. sc-CO_2 extraction showed a superior yield (1.2%) compared to traditional *n*-hexane extraction (0.4%), indicating its efficiency in extracting valuable bioactive compounds from biomass. These findings align with the principles of green chemistry, promoting the use of non-toxic solvents and reducing environmental impact. Following the sc-CO_2 step, the residual biomass undergoes SWE, a green technique exploiting water at subcritical temperatures to recover hydrophilic compounds like carbohydrates, proteins, and phenolic compounds. The cascade of processes ensures that nearly all valuable components are extracted from the biomass, maximizing resource efficiency while minimizing waste, which further aligns with the principles of waste valorization.

By coupling these two methods, the study demonstrates a comprehensive, zero-waste valorization strategy where each stage of the cascade is tailored to extract specific fractions of bioactive compounds, thereby addressing environmental sustainability. This integration not only reduces the need for harsh solvents but also enhances the overall extraction efficiency, reinforcing the concept of a sustainable biorefinery model where all aspects of a wasted biomass are utilized and emphasizing the synergy between advanced extraction technologies and environmental sustainability.

It should be mentioned that the industrial application of this approach presents several challenges. The scalability of both sc-CO_2 and SWE technologies remains a major limitation, as these processes often require high capital investment and energy input. Moreover, the specific conditions required to achieve optimal yields for different compounds might not always translate well to industrial scales. Further research is required to overcome the economic and technical challenges of large-scale implementation, along with conducting a life cycle assessment to more accurately evaluate the environmental sustainability of the proposed cascade approach.

CRediT authorship contribution statement

Alessio Massironi: Writing – original draft, Visualization, Investigation, Data curation. **Pamela Freire De Moura Pereira:** Writing – original draft, Validation, Investigation, Data curation. **Luisella Verotta:** Writing – review & editing, Project administration, Funding acquisition. **Amparo Jiménez-Quero:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Stefania Marzorati:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2024.122843.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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