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ORIGINAL PAPER

High-pressure homogenization-assisted extraction of bioactive compounds from *Ruta chalepensis*

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Abstract

High-pressure homogenization (HPH) was investigated to promote the extraction in water of bioactive molecules from *Ruta chalepensis*, a medicinal plant widely used in folk medicine. Aqueous suspensions (5% wt) of the pre-milled plant were treated by high-shear mixing (HSM), followed by HPH at 100 MPa for up to 10 passes. A considerable decrease in the size of the suspended particles was observed when applying HPH, which was related to cell deagglomeration and fragmentation. In contrast, no significant changes at the cellular level were observed when only maceration or HSM treatments were applied. Remarkably, HPH treatment did not significantly change the antioxidant activity of the aqueous extracts, but affected their composition: HPLC analysis revealed that HPH treatment significantly increased the content in the aqueous phase of quercetin (+ 452.7%), recovered by fractionation of the aqueous phase with ethyl acetate, and rutin (+ 29.8%), recovered with butanol. In addition, GC/MS analysis of the chloroform fractions obtained from the aqueous extracts revealed that the HPH treatment caused also a significant ($p < 0.05$) increase in γ-fagarine and chalepin of + 177% and + 1420%, respectively, whereas pteleine, skimmianine, kokusaginine, and arborinine levels were higher in the extracts obtained by maceration than the HPH-treated samples. These findings suggest that the recovery of low water-solubility compounds from *R. chalepensis*, such as rutin and quercetin, as well as of some alkaloids, such as γ-fagarine and chalepin, significantly improved by HPH-assisted extraction and associated cell disruption effect.

Keywords High-pressure homogenization · *Ruta chalepensis* · Extraction · Rutin · Quercetin · Alkaloids

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Introduction

Despite a large number of available organically-synthetized molecules with biological activity, the consumers' interest towards natural products, directly extracted from plants, has significantly increased in recent years because of their higher perceived safety and health-beneficial properties [1]. Extraction is a key step in phytochemical research to recover valuable bioactive compounds from different plant materials. Several methods of extraction have been developed, including maceration, decoction, infusion, percolation, and Soxhlet. However, some of these methods require significant heating, which can affect and degrade the thermo-sensitive compounds and cause the loss or decay of their biological activity. Furthermore, most of these methods demand high-energy consumption, high solvent to solid ratios, and long processing times, and are typically characterized by low extraction yields [1]. Recently, novel methods, able to induce the mechanical cell disruption of the plant materials and to fully unlock

the different intracellular compounds into an aqueous external medium have gained a growing interest [2]. High-pressure homogenization (HPH) is widely used in the food, cosmetic and pharmaceutical industries to produce more homogenous and stable emulsions by applying high pressure, generally ranging from 50 to 200 MPa, to reduce the particle size and improve the stability as well as the bio-availability of the product [3]. HPH is also considered as a widely employed method for cell disruption [4, 5]. During HPH treatment, the enhanced release of intracellular constituents in the external medium can be attributed to the physical disruption of the cells, which are exposed to intense fluid-mechanical stresses in the homogenization valve [4, 6]. Many studies have reported the amelioration of extraction yield of plant compounds from different vegetal materials using HPH. Xing et al. reported that HPH treatment significantly contributed to enhancing the extraction of sulforaphane (an isothiocyanate) from broccoli seeds as a function of the applied pressure, through cell disruption and enhanced mass transfer in the aqueous solvent [7]. Lycopene yield was significantly improved applying HPH treatment to tomato peel aqueous suspensions [4]. Antioxidant compounds were recovered in the aqueous phase when treating by microfluidization corn bran [8] and wheat bran [9].

Ruta chalepensis is a medicinal plant widely used in folk medicine. The plant is characterized by a chemical composition rich in bioactive compounds of therapeutic and food interest, including several phenolics, alkaloids, and volatile compounds [10]. Among these compounds, especially abundant are some with a widely reckoned biological activity, such as quercetin [11] and rutin [12]. Quercetin and rutin are considered important dietary flavonoids with several beneficial effects on human health, primarily for their antioxidant activity, as well as for their contribution to preventing and managing several diseases, including cancer, cardiovascular, and inflammatory disorders [13, 14]. Moreover, these two compounds can be used in the preservation of food as anti-bacterial agents and it was reported that the association of quercetin and rutin resulted in the enhancement of the antibacterial effect [15]. Alkaloids are another class of bioactive compounds of *R. chalepensis* principally composed of furoquiolines and acridones [16], characterized by a variety of pharmacological activities, especially antimicrobial activity [17] and the capacity to inhibit certain enzymes [18]. Therefore, the enrichment of the extracts with these compounds can be of high applicative interest.

This work aimed to investigate the HPH-assisted extraction of valuable bioactive compounds from *R. chalepensis*, especially addressing the extraction of quercetin and rutin as well as its alkaloids, in comparison with conventional maceration or high-shear mixing, not only in terms of extraction yields but also of selectivity towards the different intracellular metabolites of *R. chalepensis*.

Experimental

Chemicals

2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate,2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, bovine serum albumin (BSA), D-glucose, copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$), and Folin-Ciocalteu reactant, ethyl methanol, ethyl acetate, and n-butanol (analytic grade) were all purchased from Sigma Aldrich (Merck Life Science s.r.l., Milano, Italy).

Plant material

Ruta chalepensis L. aerial parts were collected in June 2018 from the Adekar region (North of Bejaia, Algeria: 36° 41`00``North 4° 40`00`` East, Height 1092 m). Plant identification was performed by referring to a previously reported description [19]. The plant was cleaned from dust and soil, air-dried, and ground using a laboratory blender (MF 10 B, IKA Labortechnik, IKA®-Werke GmbH & Co. KG, Staufen, Germany) and then passed through a sieve (Endecotts LTD, London, UK) to obtain a mean particle size lower than 250 µm.

Cell disruption pretreatment

Water maceration

As a control, 10 g of the plant powder was subjected to maceration in 190 g of bidistilled water at room temperature, under continuous agitation in a magnetic stirrer for 1 h. The suspension was left to sediment overnight at 4 °C, and subsequently, the supernatant was recovered in a refrigerated centrifuge (6000 rpm or 5289 ×g for 10 min at 4 °C, using an ALC PK 130R model, ALC International s.r.l., Milan, Italy) and then filtered through a filter paper. The aqueous supernatant was submitted to liquid–liquid fractionation with organic solvents.

High-shear mixing and high-pressure homogenization treatment

10 g of the powder was suspended in 190 g of bidistilled water by high-shear mixing (HSM) using an Ultra Turrax T25 (IKA Labortechnik), equipped with an S25 N18 G rotor and operated at 10,000 rpm. Samples of the homogenate were taken each 2.5 min for a total of 30 min,

to measure the antioxidant activity of the supernatant obtained after centrifugation of the samples at 5.289 ×g for 10 min. The mean particle size of the suspended particles was also determined for each sample.

The HSM suspensions after 5 min of processing (as discussed in the results, no significant effect on mean particle size was observed after 5 min of HSM, which served mainly to suspend the particles prior to HPH) were, subsequently, submitted to HPH processing. To avoid the blockage of the homogenization valve, the HSM suspensions were preliminarily sieved with a mesh size of 600 µm (Endecotts LTD), which removed only a small fraction of the solids from the suspension (the final concentration always remained > 4.9 wt %). HPH was carried out using an orifice valve assembly (orifice diameter of 150 µm) at 100 MPa for up to 10 passes. After each pass, the suspensions were cooled down in a tube-in-tube heat exchanger set at 5 °C, to ensure that the product temperature was always below 25 °C. Samples were withdrawn after 1, 2, 3, 4, 5, 7, and 10 passes, to analyze the effect also of HPH processing on the particle size distribution and antioxidant activity of the supernatant, in comparison with maceration and HSM. The suspensions obtained after HSM or after 10 HPH passes were left to sediment overnight at 4 °C and then centrifuged (5.289 ×g for 10 min at 4 °C), with the resulting supernatant being collected and submitted to a liquid–liquid fractionation using different organic solvents.

Particle size distribution determination

Particle size distributions (PSD) of *R. chalepensis* suspensions were analyzed by laser diffraction at 25 °C, using a Master Sizer 2000 particle size analyzer (Malvern Panalytical Ltd, Malvern, UK). The volume-weighted mean diameter D[4,3] and surface weighted mean diameter D[3,2], together with the median diameter d(0,5) of the distribution, as previously defined [5], were recorded for each sample and plotted against time. The parameters used in the determination of the PSD were the properties of water at 25 °C (refraction index = 1.33), which was used as a dispersant medium. The particle size distribution in the supernatant obtained from suspensions subjected to HPH treatment or water maceration was determined at 25 °C using a high-performance particle sizer based on dynamic light scattering (HPPS, Malvern Panalytical Ltd), associated to a dispersion Technology Software version 4.20 (Malvern Panalytical Ltd). The hydrodynamic diameter d_H (also known as z-average diameter) and the polydispersity index (PdI) were recorded for each sample.

Liquid–liquid fractionation

The obtained aqueous extracts (supernatant after centrifugation) from the maceration and HPH were subjected to a liquid–liquid fractionation in a separatory funnel, using first ethyl acetate and then butanol as organic solvents (150 mL of aqueous phase extracted with 50 mL of the organic phase, repeated 3 times in sequence for each solvent). For the alkaloid analysis, the aqueous supernatant was, instead, extracted using chloroform as a solvent (150 mL of aqueous phase extracted with 50 mL of chloroform three times). The different fractions were subjected to spectrophotometric and HPLC analysis of the phenolic compounds, whereas the chloroform fractions were subjected also to alkaloid analysis by GC/MS. Solvents were removed in a rotary evaporator under reduced pressure (Rotary evaporator R-114, Buchi Italia s.r.l, Milan, Italy) to recover different fractions in powder form.

Antioxidant activity

The antioxidant activity of the samples was evaluated using the ferric reducing antioxidant power (FRAP), according to a previously described method [20], with some modifications. FRAP reagent was freshly prepared by mixing, at a ratio of 10:1:1, 300 mM acetate buffer (pH 3.6, made of 3.1 g of sodium acetate and 16 mL of glacial acetic acid dissolved in 1 L of distilled water), 20 mM ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and 10 mM of 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM hydrochloric acid (HCl). 2.5 mL of the reagent was mixed with 500 µL of the sample (supernatant from centrifugation of aqueous suspensions) and incubated at room temperature for 10 min before the absorbance is read at 593 nm using a V-650 spectrophotometer (Jasco Inc., Easton, MD, USA). Ascorbic acid was used to obtain a standard curve so that the results could be expressed as µg ascorbic acid equivalent (AAE)/mg of extract.

Total solid, total phenolics, proteins and polysaccharides content of the aqueous extracts

Total solid or dry matter in supernatants was determined by taking 5 g of the supernatant in a cup, which had been previously dried in an oven (100 °C for 1 h) and weighted. Samples were placed in the oven (Model T6, Heraeus, Hanau, Germany) at 105 °C for 4 h until complete evaporation of the water. After drying, the cups were put in a desiccator for 20 to 30 min to eliminate residual moisture and then weighted again. The total solid content was determined as the weight difference between cups without supernatant and cups with supernatant after drying. Results were expressed as mg/g of solution.

Total phenolics were determined by the Folin-Ciocalteu reagent [21]. A volume of 200 µL of the sample was mixed with 2.6 mL of water, 1 mL of sodium carbonate (7%), and 200 µL of the Folin-Ciocalteu reagent. The mixture was left to incubate for 90 min and the absorbance was then read at 745 nm. Gallic acid was used to prepare a standard calibration curve.

The protein content in the aqueous extract was determined by the Lowry method [21] with some modifications. 1 mL of the sample was mixed with 5 mL of reagent C prepared by mixing reagent A (Na_2CO_3 (2%) in NaOH (0.1 N)) and reagent B (½ volume of $CuSO_4$ (0.5%) and ½ volume $KNaC_4H_4O_6{\cdot}4H_2O$ (1%)) at the proportion of 50:1. A volume of 0.5 mL of Folin-Ciocalteu reagent (diluted at a ratio of 1:2) was then added to the mixture and incubated for 35 min in the dark. The absorbance was then read at 750 nm. BSA (Bovin Serum Albumin) dissolved in Tris–HCl buffer (60 mM added of 2% of SDS) was used to prepare the standard calibration curve.

Total carbohydrates content was determined using the modified phenol–sulfuric acid assay [22]. A volume of 200 µL of the diluted sample (1/10) was mixed with 200 µL of phenol (5% w/v) and then 1 mL of the concentrated sulfuric acid was added. The mixture was vigorously vortexed and incubated at 30 °C for 30 min in a shaker incubator (Stuart S170, Bibby Sterilin, Cole-Parmer, Stone, UK) set at 120 rpm. Absorbance was then read at 490 nm against a blank containing all the reactives without the sample being added. A standard calibration curve was prepared using D-glucose and results are expressed as mg D-glucose/g of dry weight.

Chromatographic analysis

The fractionated extracts in ethyl acetate and butanol were dried, accurately weighed, dissolved in methanol and filtered through 0.45 µm filters. Then, 5 µL of the filtrate was analyzed using a Waters HPLC (Model 1525, Waters Corporation, Milford, MA, USA) equipped with a 1525 binary HPLC pump and a photodiode array (DAD) detector. The separation was performed in a reversed-phase column (Supelco Waters spherisorb ODS-2,5um, 4.6 × 150 mm, Waters Corporation) at ambient temperature. The mobile phase was composed of solvent A (phosphoric acid 0.1%) and solvent B (methanol). The elution conditions were set as follows: 5% B, linear gradient to 80% of eluent B in 30 min, linear gradient to 80% of B in 3 min, and finally returning from 80% B to 5% B in 2 min. The flow rate was 0.8 mL/min and the detection was performed at 256 nm for rutin and 368 nm for quercetin. Compounds were identified by comparing their retention time with those of pure standards and the results for rutin and quercetin were reported as mg/g of the dry extract by calibration with the corresponding standard curves.

Chloroform fractions were analyzed for their alkaloids and coumarins composition by gas chromatography coupled with the mass spectrometry (GC/MS) according to a previously described method [23]. Samples were first dried and then dissolved in dichloromethane and injected in a GC/MS (Focus DSQ, Thermo Electron Corporation, Austin, TX, USA) equipped with an Agilent DB-5MS capillary column (30 m × 0.250 mm × 0.25 µm, Agilent Technologies, Inc., Santa Clara, CA, USA). The temperature of separation was programmed as follows: the initial temperature was set at 60 °C and maintained for 3 min. Then, it was increased to 100 °C at a rate of 3 °C/min and held for 1 min, and then at a rate of 5 °C/min to 140 °C for 1 min. Finally, the temperature was raised to 240 °C at 20 °C/min and maintained for 10 min. The injection volume was 1 µL in splitless mode.

The carrier gas was helium with a flow rate of 1 mL/min. The ion source of the MS was operated at 250 °C, the inlet temperature was 230 °C, and the X-line temperature was 250 °C. Data collection and processing were performed using the GC/MS Xcalibur Software (Thermo Electron Corporation). The identification of alkaloids and coumarins was performed by comparing their MS and retention time (R_t) with the data from the NIST library and the literature. However, no quantification of the individual alkaloids was carried out. The results are reported qualitatively as peak areas of the identified compounds and their percentage variations, for a single experiment.

Statistical analysis

Data were reported as means ± standard deviation of three measurements, unless differently indicated. Analysis of variance was performed by one-way ANOVA analysis using Graphpad prism software version 5 (GraphPad Software Inc, San Diego, CA, USA) followed by Tukey's multiple comparison test. Means are considered to be significantly different at $p < 0.05$.

Result and discussion

Effect of cell disruption treatments on *Ruta chalepensis* suspensions

The effect of HSM and HPH processing on the particle size is reported in Fig. 1. HSM treatment did not cause any significant variation in the particle size distribution of the suspensions, as shown by the values of the characteristic diameters D[4,3], D[3,2], and d(0,5), as a function of the HSM processing time. In particular, the size distribution of the HSM suspensions is very similar to that of control

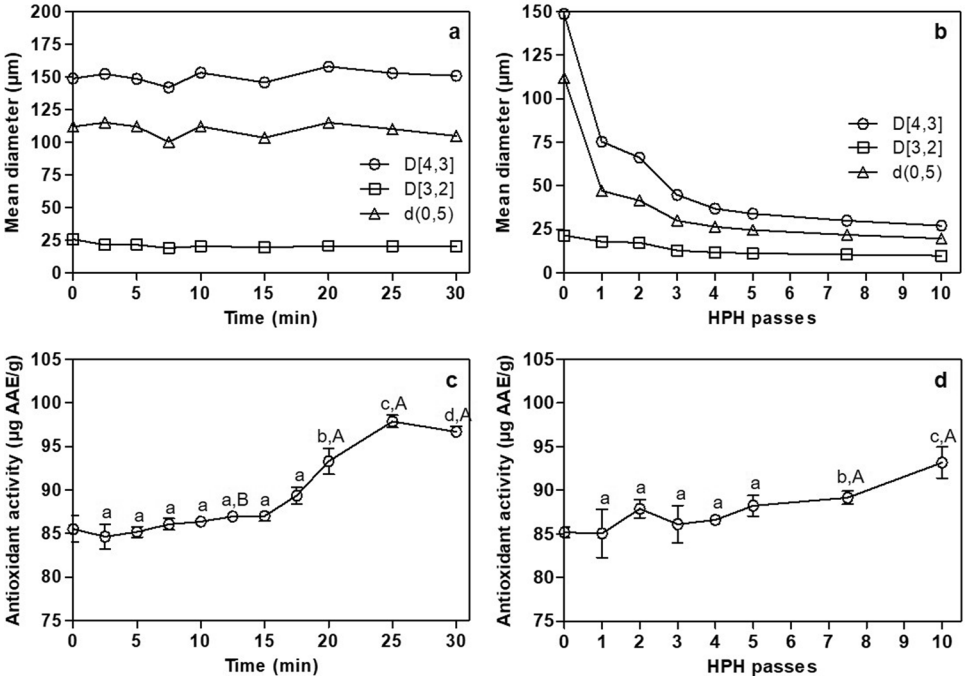

Fig. 1 Effect of **a** HSM treatment time and **b** HPH for 10 passes at 100 MPa on the mean diameters of *Ruta chalepensis* aqueous suspensions, and antioxidant activity of the supernatant of *Ruta chalepensis* aqueous suspensions after **c** HSM for 5 min and **d** HPH for 10 passes at 100 MPa. Values superscripted with different letters are significantly different ($p < 0.05$): lower case letters refer to the comparison between control (t = 0 min or 0 passes) and supernatant at each time of processing or HPH pass, upper case letters refer to the comparison between two adjacent values

(untreated) suspension. However, when the HPH treatment was applied, a significant decrease in particle size distribution was observed, as shown by the trend of the characteristic diameters as a function of the HPH passes, which could be attributed to cell disruption, as discussed in the following. In particular, most of the size reduction occurred within the first 4 passes, with the median value of the size distribution d(0,5) reaching a value of 26.4 µm and D[4,3] and D[3,2] tending towards 36.8 and 11.7 µm, respectively. The surface mean diameter D[3,2], which is more sensitive to small particles than the volume mean diameter D[4,3], changed at a lower rate than D[4,3]. After 4 passes, only small variations were observed for D[4,3] and d(0,5).

The *R. chalepensis* suspensions treated by maceration, HSM, or HPH exhibited a very different appearance (Fig. 2a), as a consequence of cell disruption, which can be clearly observed in Fig. 2b, c. The appearance of the suspensions exhibited a noticeable modification between maceration, HSM, and HPH, becoming progressively smoother and more homogeneous as treatment intensity increased. In comparison with the maceration sample, the HSM treatment showed a lower number of cell clusters, which are visible by the naked eye; however, only after HPH treatment, the cell clusters could not be distinguished by the naked eye, and a smooth and homogeneous suspension was obtained. HPH processing was responsible for the deagglomeration of the cell clusters and inducing cell walls disruption, with significant structural changes, which are evident in Fig. 2b, c. More specifically, the cellular structural organization, which can be observed for *R. chalepensis* suspensions upon maceration, and which is partly preserved after HSM treatment, was completely disrupted after HPH processing, with the formation of small cell fragments. HSM processing, in agreement with previous investigations on tomato peels suspensions, was not able to cause the disruption of plant cells, but only the deagglomeration of cell clusters [4].

Effect of cell disruption treatment on the supernatant

Supernatants from maceration, HSM, and HPH treatments were analyzed for their antioxidant activity (Fig. 1c, d), as well as for their content in proteins, carbohydrates, total phenolics, and total solids (Fig. 3c, d). Moreover, also the size distribution of the residual solids in the supernatant, evaluated in terms of hydrodynamic diameter and PdI, was determined (Fig. 3a, b).

Different processing times by HSM did not cause any significant difference in terms of antioxidant activity of the supernatant, as assessed with FRAP, in comparison with the supernatant of the control suspension (t = 0 min) until 20 min of processing, when, instead a significant increase was observed ($p < 0.05$). Remarkably, also the HPH treatment did not cause any significant increase in the antioxidant activity of the supernatant, which significantly ($p < 0.05$) increased in comparison with the control of less than 10% only after 8 and 10 passes, reaching the value of 93.2 ± 1.8 µg AAE/g of powder after 10 passes. These results suggest that *R. chalepensis* contains a significant fraction of hydrosoluble compounds, which are efficiently released in the aqueous

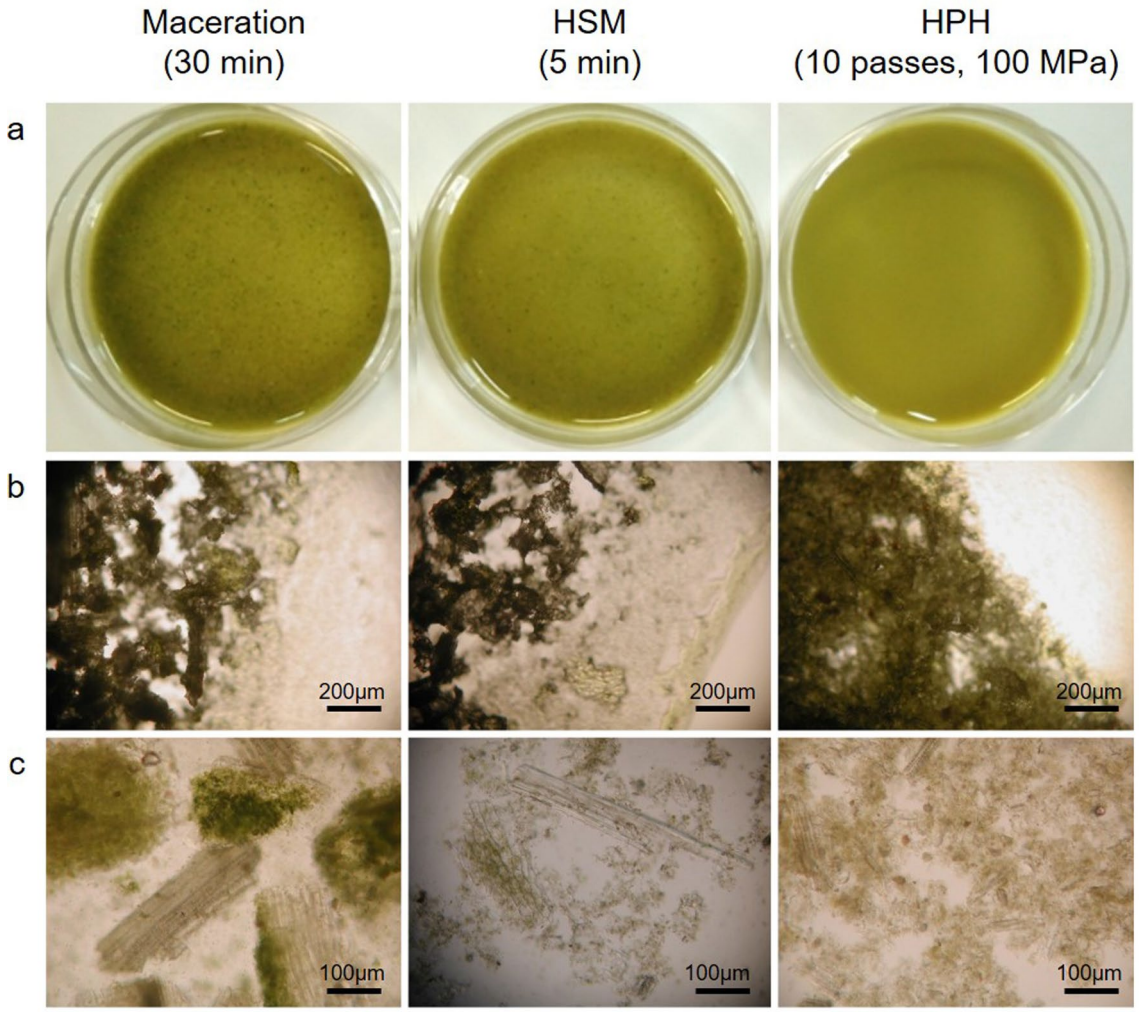

Fig. 2 Effect of maceration for 30 min, HSM for 5 min and HPH for 10 passes at 100 MPa on *Ruta chalepensis* aqueous suspensions; **a** digital photographs and **b**, **c** optical microscopy images at different magnification

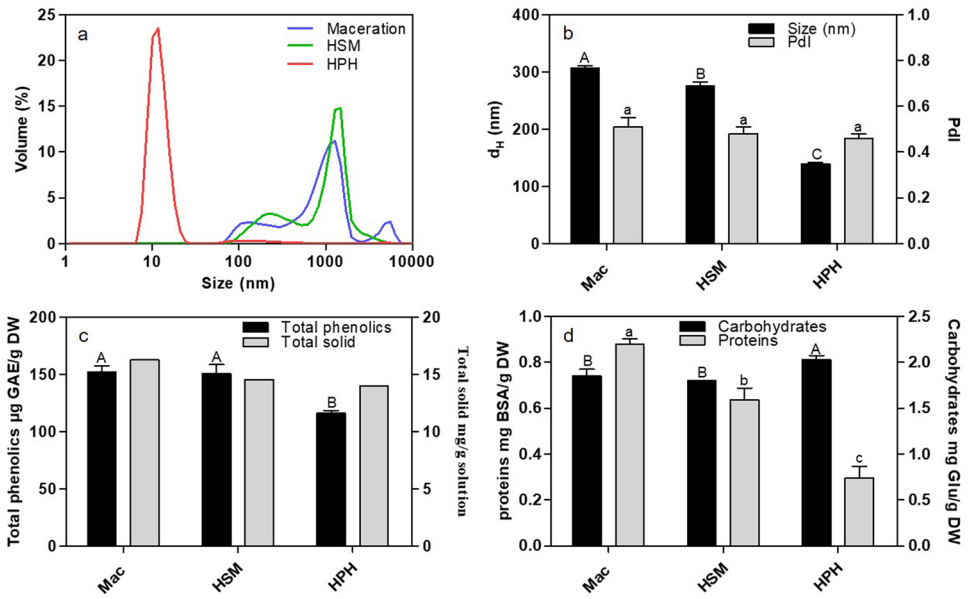

Fig. 3 Characterization of the supernatant from maceration (Mac), HSM (30 min of treatment), and HPH (10 passes at 100 MPa) in terms of particle size volume distribution (**a**), hydrodynamic diameter d_H and polydispersity index PdI (**b**), total solid and total phenolics (**c**) total proteins and total carbohydrates (**d**). Columns superscripted with different letters are significantly different ($p < 0.05$). Lower and upper case letters were used to distinguish the statistical analysis of two parameters in the same graph

phase, without the need of HSM-assisted deagglomeration or HPH-assisted cell disruption. Only at higher treatment intensities, some small effects can be observed.

It must be remarked that the centrifugation process did not cause the complete removal of all the suspended particles, but only of the larger ones (e.g. cell debris), whereas the finer ones (e.g. insoluble molecules and molecular complexes) remained in suspension. This is shown by the dynamic light scattering analysis of the supernatant, as reported in Fig. 3a. Interestingly, HPH processing caused a significant reduction in size and narrowing of the size distribution of the particles still suspended in the supernatant after centrifugation: the hydrodynamic diameter of the particles present in the supernatant for the maceration was 307.0 ± 3.5 nm and significantly decreased ($p < 0.05$) for HSM sample to 276.3 ± 6.7 nm, and further decreased for HPH to 138.7 ± 3.1 nm (Fig. 3b). This observation is in agreement with previous studies on the serum (supernatant) obtained from frozen concentrated orange juice, treated by HPH, which was significantly clearer than the serum from unprocessed juice [24], because of the smaller suspended particles obtained after HPH treatment, which are more difficult to remove from the supernatant during centrifugation. However, no significant difference was observed for PdI after HSM and HPH, in comparison to maceration (Fig. 3b).

The total solids content and total proteins decreased when HSM and HPH were applied (Fig. 3c, d). In contrast, no statistically significant difference was recorded between the maceration and HSM for total phenolics (151.97 ± 5.27 and 150.76 ± 7.81, respectively), while for HPH a decrease was observed (Fig. 3c). This is in agreement with previous studies, where the observed decrease in total phenolics upon microfluidization treatment of corn bran was attributed to the fact that the high-energy dispersion of free phenolic compounds in the water phase might cause their degradation [8]. However, this explanation is not fully convincing for the results of Fig. 3, because of the concurrent increase in antioxidant activity, and, as shown in the following section, of the concentration of water-insoluble bioactive compounds, such as quercetin and rutin upon HPH treatment. It is likely that HPH processing, promoting selectively the release of water-insoluble compounds, causes a significant change the distribution in aqueous phase of bioactives, in comparison with maceration. Folin-Ciocalteu method used for the determination of total phenolics is non-specific, and therefore, the resulting value might be significantly affected by the composition of the extracts, as well as by the presence of other molecules in the sample. Further studies are required to better clarify this aspect.

The highest value for carbohydrates was observed for HPH (0.81 ± 0.02 mg/g of powder) while no significant difference was obtained between maceration and HSM (Fig. 3d). Previous studies showed that the application of HPH improved the extraction yield of proteins from peanut [25] and microalgae [26]. However, the present work reported a decrease in proteins yield when applying HSM and HPH, which can be attributed to the denaturation of proteins, therefore reducing their solubility in the aqueous medium and causing their precipitation with centrifugation. Desrumaux and Marcand reported the occurrence of structural conformation changes in the whey proteins when used to stabilize sunflower oil emulsions after application of a HPH treatment [27].

Identification of the main compounds in the different extract fractions

HPLC analysis of ethyl acetate and butanol fractions was performed to determine their content in quercetin and rutin, with the resulting chromatograms reported in Fig. 4, and the quantified values against quercetin and rutin standards in Fig. 5. Quercetin was recovered in significantly higher concentrations by ethyl acetate fractionation, due to its aglycone structure. In contrast, rutin, presenting a glycosylated structure, was better recovered by butanol fractionation. When comparing the effect of maceration and HPH on the recovery of quercetin and rutin, it can be observed that, in butanol, rutin increased more than 29.8% (from 160.82 ± 2.54 to 208.70 ± 1.13 mg/g of dry extract) and that, in ethyl acetate, quercetin increased more than 452.7% (from 2.98 ± 0.21% to 16.47 ± 0.28 mg/g of dry extract). Loizzo et al. reported an amount of 266.7 mg/g dry extract in the methanolic extract for rutin and 12.4 mg/g dry extract for quercetin [11], which are close to the amounts reported by the present work. Similarly, Ntalli et al. reported rutin as the most abundant constituent of methanolic extracts from *R. chalepensis* [28]. The HPLC chromatograms (Fig. 4) clearly show the differences in peak areas between ethyl acetate and butanol as well as between maceration and HPH. Quercetin is one of the most representative flavonoids found in fruits, vegetables, and medicinal plants. Naturally, quercetin occurs in a glycosylated form associated with sugar moieties such as rutinose to give rutin [29]. In fact, quercetin, in its pure form, is scarcely soluble in aqueous media (0.06 mg/mL) [30]. Its presence in the aqueous extracts, which at 5% concentration of solid reaches 1.0 and 1.2 mg/mL, respectively for maceration and HPH, is two orders of magnitude higher than quercetin solubility and may be explained by its molecular interaction with other compounds, such as proteins and polysaccharides, that contribute to maintaining it in suspension in water. A similar observation can be made for rutin, which is only slightly more soluble than quercetin (0.125 mg/mL) [31], but reaches concentrations in the aqueous phase as high as 7.2 and 13.7 mg/mL for maceration and HPH, respectively. It must also be highlighted that HPH treatment might contribute to enhancing the dispersibility in

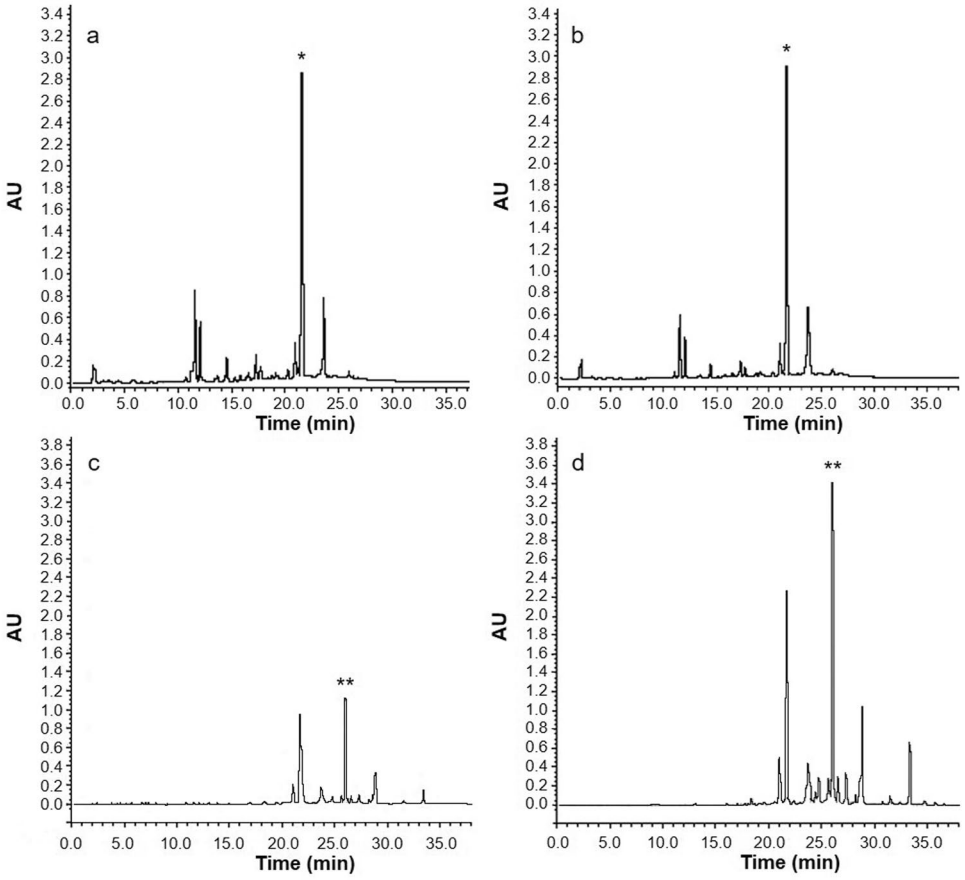

Fig. 4 HPLC profile in the butanol fraction (**a**, **b**) and in the ethyl acetate fraction (**c**, **d**) obtained from the aqueous supernatant from maceration (**a**, **c**) and HPH treatment (**b**, **d**). The main identified peaks correspond to rutin (*) in the butanol fraction (**a**, **b**) and quercetin (**) in the ethyl acetate fraction (**c**, **d**)

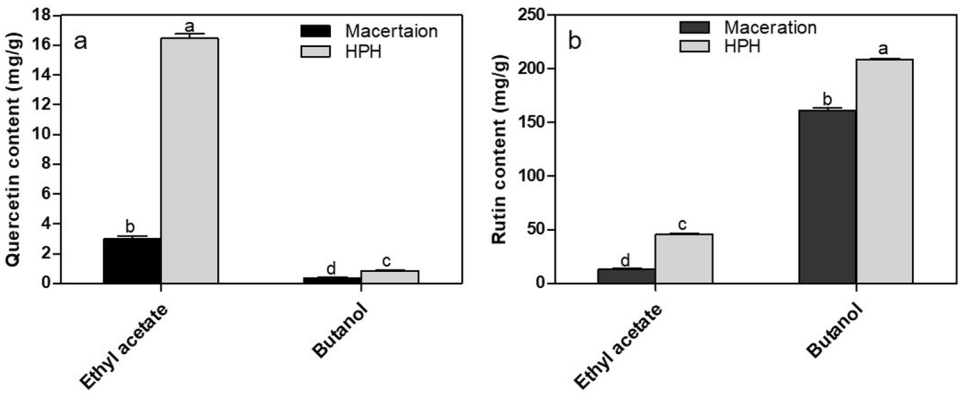

Fig. 5 Effect of HPH treatment on the concentration of (**a**) quercetin and (**b**) rutin in the dry extracts obtained from ethyl acetate and butanol fractionations, compared to maceration

water of insoluble crystals, such as quercetin, by inducing a loss of crystallinity as a consequence of the applied mechanical friction and stresses [32], especially if in the presence of emulsifying molecules, which are naturally available in plant cells.

Chloroform fractions were obtained for GC/MS analysis and addressed to the identification of five alkaloids and one coumarin, as shown in the chromatograms in Figure S1 of Supplementary Material. More specifically, four furoquinolines [γ-fagarine (1), pteleine (2), skimmianine (3), and kokusaginine (4)], one acridone [arborinine (6)] and a furocoumarin [chalepin (5)] were identified in the chloroform fractions from both maceration and HPH treatments. Kokusaginine and skimmianine constituted the predominant alkaloids in both chloroform fractions, which is in agreement with prior findings [33]. Kacem et al. previously reported the presence of these compounds in the aqueous extract [34]. When they occur in free form, alkaloids are basic and not soluble in water [35]. However, in plants, alkaloids usually occur as salts (citrates, tartrates, meconates, isobutyrates,

Table 1 Main alkaloids compounds identified in chloroform fraction obtained from maceration and HPH extraction, numbered in agreement with Figure S1 of Supplementary Material, together with their chemical formula, molecular weight, as well as GC–MS retention time, peak area and percent variation of peak area of HPH treatment with respect to maceration

N	Compound	Formula	M_w	R_t (min)	Peak area		Variation (%)
					Maceration	HPH	
1	γ-Fagarine	$C_{13}H_{13}NO_3$	229.06	20.25	1,947,012	5,391,045	+ 176.9
2	Pteleine	$C_{13}H_{13}NO_3$	229.07	20.45	18,304,672	14,050,130	− 23.2
3	Skimmianine	$C_{14}H_{15}NO_3$	259.07	21.76	14,744,381	13,365,364	− 9.4
4	Kokusaginine	$C_{14}H_{15}NO_3$	259.05	22.55	122,870,338	92,798,025	− 24.5
5	Chalepin	$C_{19}H_{22}O_4$	314.13	24.22	2,144,304	32,575,294	+ 1419.2
6	Arborinine	$C_{16}H_{15}NO$	285.08	26.69	12,646,678	10,447,707	− 17.4

R_t Retention time, M_w molecular weight (g/mol)

and benzoates) or combined with tannins, and, therefore, are soluble in water [36], which can explain their presence in the aqueous extract. Table 1 reports, together with the distinguishing molecular characteristics of the identified compounds, also the percentage of variation of their peak areas (V), calculated using the following formula:

$$V = \left(A_{HPH} - A_{Mac}\right)/A_{Mac} \times 100\%,$$

where A_{Mac} and A_{HPH} are the peak areas of the compounds recovered in chloroform from the supernatant of maceration and HPH-treated suspensions, respectively.

γ-Fagarine and chalepin were more abundant in the chloroform fraction from HPH extraction than from the maceration extraction, showing a significant difference in the peak area (V) of + 177% and + 1420% respectively. In contrast, in the case of the other alkaloids (pteleine, skimmianine, kokusaginine, and arborinine), a higher concentration was observed in the maceration extracts than in the HPH ones.

In summary, the use of HPH as a pretreatment to assist the extraction of bioactive compounds from *R. chalepensis*, applied to a 5% wt aqueous suspension of *R. chalepensis*, caused a significant reduction of the particle size in the aqueous suspension, which can be associated with cell disruption and size reduction of the resulting cell debris. This disruptive effect is not observed during maceration or HSM treatments. Remarkably, the HPH pretreatment had a significant effect also on the composition of the extracts, especially for what concerns the amount of quercetin and rutin that can be recovered upon fractionation in ethyl acetate and butanol, respectively, of the aqueous phase recovered through the centrifugation of the HPH-treated suspensions. More specifically, quercetin concentration increased by about 452.7% upon HPH treatment, whereas the concentration of rutin showed an increase of about 29.8%. In addition, also the alkaloids profile in the aqueous extract changed, with a richer composition observed for γ-fagarine and chalepin in HPH extracts than in maceration extracts, and lower concentrations for pteleine, skimmianine, kokusaginine, and arborinine.

The main finding of this work is that HPH treatment enables the extraction with high yields of different compounds characterized by low water solubilities, such as quercetin, rutin, and chalepin, from *R. chalepensis*, using only water as extraction solvent. Despite these compounds are scarcely soluble in water, they are likely extracted as molecular complexes with proteins and polysaccharides, acting as dispersants and carriers. Subsequently, these compounds can be recovered through liquid–liquid fractionation, using suitable organic solvents, without contaminating with said solvents the *R. chalepensis* suspended solids, which, instead, can be disposed of as an uncontaminated plant residue, which has been subjected only to mechanical treatment. Moreover, the solvent extraction is not necessary, if the total extracts are desired, as quercetin, rutin, and chalepin are well-dispersed and stabilized at high concentrations in the aqueous supernatant.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Lynda Gali and Francesco Donsì. The first draft of the manuscript was written by Lynda Gali and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Most of the data not explicitly presented are available in the Supplementary Material. The remaining are available upon request (fdonsi@unisa.it).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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