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Functional Properties of *Punica granatum* L. Juice Clarified by Hollow Fiber Membranes

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Abstract: There is currently much interest in pomegranate juice because of the high content of phenolic compounds. Moreover, the interest in the separation of bioactive compounds from natural sources has remarkably grown. In this work, for the first time, the *Punica granatum* L. (pomegranate) juice—clarified by using polyvinylidene fluoride (PVDF) and polysulfone (PSU) hollow fiber (HF) membranes prepared in the laboratory—was screened for its antioxidant properties by using different in vitro assays, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Ferric Reducing Antioxidant Power (FRAP), and β-carotene bleaching tests, and for its potential inhibitory activity of the carbohydrate-hydrolysing enzymes, α-amylase and α-glucosidase. The effects of clarification on quality characteristics of the juice were also investigated in terms of total phenols, flavonoids, anthocyanins, and ascorbic acid. Experimental results indicated that PVDF membranes presented a lower retention towards healthy phytochemicals in comparison to PSU membranes. Accordingly, the juice clarified with PVDF membranes showed the best antioxidant activity. Moreover, the treatment with PVDF membranes produced a clarified juice with 2.9-times fold higher α-amylase inhibitory activity in comparison to PSU (IC_{50} value of 75.86 vs. 221.31 μg/mL, respectively). The same trend was observed using an α-glucosidase inhibition test. These results highlight the great potential of the clarified juice as a source of functional constituents.

Keywords: pomegranate; dietary antioxidants; hypoglycaemic effects; ultrafiltration (UF); functional drink

1. Introduction

Punica granatum L. (pomegranate) juice is becoming increasingly popular due to the attribution of important biological properties [1] Since ancient times, pomegranate fruit has been an economically important plant in the food and pharmaceutical markets [2]. Along with other fruits—namely blueberry, bilberry, elderberry and cranberry—pomegranate has been elected as a "superfruit" principally for its significant antioxidant potential [3]. Pomegranate is currently ranked 18th in terms of global annual fruit consumption [4]. The widespread public knowledge of the health attributes has led to a large rise in the demand for this fruit and its by-products during recent years [5]. Among pomegranate-derived products, juice is one of the most popular drinks in the super juice category, and is consumed around the world because of its pleasant and unique aroma, flavour, and colour.

Pomegranate juice provides a simple way to consume healthy active nutrients that have recently been recognized for their antioxidant, anticarcinogenic, and antimicrobial activity [6]. Furthermore, it

has been recognized that its consumption is helpful in fighting different pathologies, such as Alzheimer's disease, type 2 diabetes, and heart disease. Moreover, the utilisation of pomegranate juice or juice derivatives as food colorants and flavour enhancers further has further increased its production. A perusal of literature revealed the presence of more than 120 different phytochemicals in pomegranate fruit belonging to the polyphenols class (anthocyanins, gallotannins, ellagitannins, hydroxycinnamic acids, hydroxybenzoic acids, and gallagyl esters) [7].

In recent years, antioxidants have gained a great interest because of their potential as prophylactic and therapeutic agents in many diseases. Consequently, the global market of antioxidants is increasing rapidly, because of the increased health risk in a constantly polluting environment. These agents also have pharmaceutical and/or nutraceutical applications, leading to the development of research at industrial and academic levels to explore these molecules and their analogues. For these reasons, there is great interest in the separation, purification, and recovery of antioxidant compounds from natural sources.

Epidemiological studies show that consumption of fruits with high phenolic content reduces the risk of several diseases [8]. In the past few years, there has been an increasing interest in determining relevant dietary sources of antioxidant phenolics. Thus, red fruit juices such as grape and different berry juices have received attention due to their antioxidant activity.

High levels of free radicals and the coincident decline of endogenous antioxidant defence mechanisms can lead to damaged cells, increased lipid peroxidation, and the development of insulin resistance (resulting either from the body's ineffective use of insulin, a low level of insulin production in pancreatic β-cells, or peripheral insulin resistance), that characterizes type 2 diabetes and the development of diabetic complications [9]. For these reasons, molecules with antioxidant potential are useful for the adequate maintenance of oxidative levels in the blood. Recently, a review article described the potential antidiabetic effect of pomegranate by reporting both basic and clinical research studies related to the effects of pomegranate and some of its bioactive constituents on biochemical and metabolic parameters linked to type 2 diabetes [10].

The appearance of a product influences the choice of consumers in various ways. Consumers have a strong preference for clear juices. Based on the needs of the market, the food industry is always looking for new methods that can enrich drinks in healthy products and at the same time provide a clarified product that has good organoleptic properties [11].

Conventional methods of producing clarified juice involve many steps, such as enzymatic treatment, cooling, flocculation, decantation, centrifugation, and filtration. These processes are characterized by several drawbacks in terms of prolonged processing times, significant juice loss, and environmental problems due to the disposal of fining agents. Therefore, the search for new approaches to the clarification of pomegranate juice is very active. The use of chitosan as a clarifying agent in the production of pomegranate juice was recently evaluated, and its effects on quality characteristics of the juice were investigated [12]. Membrane processes, such as microfiltration (MF) and ultrafiltration (UF), have proven to be an attractive substitute to conventional clarification procedures. Their main advantages are in terms of (a) increased juice yield; (b) possibility to operate in a single step, reducing working times; (c) reduction in enzyme utilization; (d) possibility to operate at room temperature, preserving juice freshness, aroma and nutritional value; (e) easy cleaning and maintenance of the equipment; (f) possibility of avoiding the use of gelatines, adsorbents and other filtration aids; and (g) improvement of the juice taste through the removal of tannins [13,14]. Recently, the clarification of pomegranate juice by hollow fiber (HF) membranes was investigated [15] by comparing the performance of poly(ether ether) ketone (PEEKWC), and polysulfone (PSU) membranes. In another study, the clarification of pomegranate juice with polyvinylidene fluoride (PVDF) membranes was evaluated with particular emphasis on the fouling mechanism occurring during the clarification process [16].

This work aimed at investigating the biological properties of pomegranate juice clarified by membrane filtration. Experimental activities addressed: (a) the preparation and characterisation

of PVDF and PSU hollow fiber membranes and their use for the clarification of the juice in selected operating conditions; (b) the evaluation of phytochemicals content (phenols, flavonoids, anthocyanins, and ascorbic acid content); (c) the analysis of antioxidant activity of clarified juice by using four in vitro assays, namely 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and β-carotene bleaching tests; and (d) the assessment of the hypoglycaemic activity of the treated juice through the inhibition of carbohydrate-hydrolysing enzymes α-amylase and α-glucosidase.

2. Materials and Methods

2.1. Chemicals and Reagents

Solvay Advanced Polymers kindly provided PVDF Solef® 6010, Sigma-Aldrich (Milan, Italy) supplied PSU and BASF supplied PVP Luviskol K-17. All the other chemicals were of analytical grade and used without further purification. *N*-Dimethylacetamide (DMA), *N*,*N*-dimethylformamide (DMF), ethanol, methanol, dimethyl sulfoxide (DMSO), and potassium iodide were obtained from VWR International s.r.l. (Milan, Italy). Potato starch, sodium phosphate, sodium chloride, α-amylase from porcine pancreas (EC 3.2.1.1), α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), maltose, sodium acetate, sodium potassium tartrate, 3,5-dinitrosalicylic acid, *o*-dianisidine color reagent (DIAN), glucose oxidase peroxidase enzyme solution (PGO), Folin-Ciocalteau reagent, cyanidin 3-glucoside, quercetin, chlorogenic acid, ascorbic acid, butylhydroxytoluene (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, Tween 20, β-carotene, and $AlCl_3$ were obtained from Sigma-Aldrich S.p.a. (Milan, Italy). Acarbose was obtained from Serva (Heidelberg, Germany).

2.2. Juice Extraction

Pomegranate (*Punica granatum* L.) fruits were purchased in October 2013 from a local market in Cosenza (Calabria, Italy). Fruits were washed and manually cut-up. The juice was extracted by using an electric squeezer and pre-filtered using a mesh filter, obtaining a red-deep color extract. The juice was then stored in a refrigerator (−17 °C) until processing.

2.3. Membrane Preparation

Polyvinylpirrolidone PVP K-17 was added to the respective solvent (DMF for PVDF and DMA for PSU) in a glass flask at the appropriate temperature (115 °C for PVDF and 50 °C for PSU) under constant stirring. Then, the polymer was added to the mixture and mixed until a homogeneous solution was obtained. The solution was left to degas overnight before spinning. The HF membranes were prepared by non-solvent induced phase separation (NIPS) technique by extruding the polymer solution, pressurised with pure nitrogen (N_2) through a spinneret, which had an inner diameter (I.D.) of 600 μm and an outer diameter (O.D.) of 1600 μm. During the extrusion of the polymer solution, the bore fluid (DMF 45 wt% for PVDF and DMA 65 wt% for PSU) was fed by means of a peristaltic pump inside the inner tube of the spinneret. Then, the extruded polymer solution was left to fall in a rotating water bath where the fibers were collected after complete coagulation. For the PVDF HF, a cylindrical humidity chamber was mounted between the spinneret and the coagulation bath creating a vapor atmosphere with a relative humidity of about 80%. Table 1 reports the composition of the polymer solution and the spinning conditions for both types of fibers. The produced membranes were then rinsed in hot water (50 °C) at least 3 times and they were subsequently treated with a solution of sodium hypochlorite 4000 ppm buffered to pH 7. Finally, the fibers were immersed in a glycerol aqueous solution (40 wt%) for 24 h in order to preserve their porous structure. The HF were finally dried at room temperature for 48 h. For the water and juice filtration tests, HF were assembled in glass modules with a length of 20 cm and fixed at each extremity with epoxy glue. Each module was equipped with three fibers.

Table 1. Composition of the polymer solution and spinning conditions.

HF	PVDF	PSU
Polymer	PVDF 6010 (25 wt%)	PSU (20 wt%)
Additive	PVP K 17 (35 wt%)	PVP K 17 (15 wt %)
Solvent	DMF (40 wt%)	DMA (65 wt%)
Bore fluid	DMF (45 wt%)	DMA (30 wt%)
Coagulation bath	Water	Water
T of the polymer solution (°C)	115	50
Air gap (cm)	23	25.5
Humidity	Yes	No
Polymer spinning rate (g/min)	9.5	9.2
Nitrogen pressure (bar)	4	0.5

2.4. Characterization Tests

Investigation of HF cross-section morphology was carried out by scanning electron microscopy (SEM) (Zeiss, EVO MA10, Oberkochen, Germany). The cross-sections were prepared by freeze fracturing them during immersion in liquid nitrogen in order to get a clear fracture. All samples were sputter-coated with gold prior to analysis. Bubble point and mean pore size were measured by using a capillary flow porometer (CFP 1500 AEXL, PMI porous materials Inc., Ithaca, NY, USA). The samples were prepared by soaking them in a low surface tension (16 dynes/cm) wetting liquid (Fluorinert ® FC-40, Sigma-Aldrich, Milan, Italy) and the measures were carried out according to the wet up/dry up method. Membrane porosity (ε_m) was calculated by a gravimetric method, and it was calculated according to the equation described elsewhere [17]. Mechanical properties (Young's modulus and elongation at break) of the produced HF were measured by a ZWICK/ROELL Z 2.5 (Zwick/Roell, Ulm, Germany) instrument. For each type of fiber, five samples of 6 cm were cut and stretched at the constant rate of 5 mm/min up to the breaking point of the sample. Contact angle was measured using ultrapure water by the method of sessile drop using a CAM200 instrument (KSV Instrument LTD, Helsinki, Finland). For each sample, at least 10 measurements were taken and the average was then calculated. Contact angle was measured on the inner side of the fibers. Water permeability was evaluated after washing the HF, mounted in the proper module, with hot distilled water (40 °C) for 30 min at 1 bar in order to remove the glycerol. The permeability was measured by feeding distilled water through the fibers at 25 °C and at different transmembrane pressures (TMP) (from 0.3 to 1.2 bar) following the inside-out configuration with a cross-flow unit.

2.5. Juice Clarification

Experimental runs were performed according to the total recycle configuration (in which both permeate and retentate streams were recirculated back to the feed tank) at different TMP values and at a temperature of 25 °C by using the same setup applied for water permeability determination.

The TMP was varied from 0.3 to 1.2 bar with a constant feed flow rate of 30 L/h. This approach allowed the evaluation of the effect of TMP on the permeate flux and the identification of the TMP limit value, beyond which the fouling phenomenon caused a significant decline of permeate flux. The raw juice was then clarified according to a batch concentration configuration in which the retentate was recirculated back to the feed tank, while the permeate was continuously collected up. The clarification process was operated at a temperature of 25 °C, a feed flow rate of 30 L/h, and a TMP of 0.6 bar.

After the filtration of pomegranate juice, HF membranes were washed with distilled water at 40 °C for 30 min at the TMP of 1 bar in order to remove the reversible polarization effect. Afterwards, an enzymatic cleaning, using a 1.0 wt % Ultrasil solution, was flushed through the fibers for 30 min at 40 °C. At the end of the enzymatic cleaning, a final rinse of the fibers with distilled water was carried out. The water permeability value was then recorded again and compared with the initial one.

2.6. pH, Suspended Solids and Soluble Solids Measurements

The pH was measured with a digital pH meter (PC 2700, Eutech Instruments, Landsmeer, The Netherlands). The suspended solid content was measured by centrifuging the samples for 20 min at 2000 rpm. The weight of suspended solids was then registered after the removal of the supernatant and expressed in relation of the total juice (wt%). The soluble solids content was measured with a refractometer (ATAGO CO., LTD, Kumamoto, Japan) at 25 °C and expressed in Brix.

2.7. Total Phenols Content

Total phenol content (TPC) of pomegranate samples was determined using the Folin-Ciocalteau method at 765 nm (UV-Vis spectrophotometer, Jenway, Staffordshire, UK) with chlorogenic acid as reference [18]. The total phenol content was expressed as chlorogenic acid equivalents in mg/L. Results were expressed as mean values of three measurements.

2.8. Total Flavonoids Content

The flavonoids content was determined spectrophotometrically as reported elsewhere [19]. Absorbance was read at 510 nm. The levels of total flavonoid content were determined in triplicate and expressed as quercetin equivalents in mg/L.

2.9. Total Anthocyanins Content

Total monomeric anthocyanin content was determined using a spectrophotometric pH differential protocol described by Giusti and Wrolstad [20]. Briefly, 0.5 mL of the extract was mixed with (a) 3.5 mL of potassium chloride buffer (0.025 M, pH 1); and (b) 3.5 mL of sodium acetate buffer (0.025 M, pH 4.5). After 15 min, the raw absorbance of each solution was measured at 510 and 700 nm. Results are expressed as mg of cyanidin 3-glucoside equivalents/L.

2.10. Ascorbic Acid Content

Ascorbic acid content was determined according to the method of Klein and Perry [21]. Results were expressed as mg per 100 mL of juice.

2.11. In Vitro Antioxidant Activities

2.11.1. DPPH (2,2-diphenil-1-picrylhydrazyl) Test

In DPPH test, a mixture of 0.25 mM DPPH and clarified juice was prepared and left at room temperature for 30 min [22]. The absorbance was read at 517 nm. The DPPH radicals scavenging activity (SA) was calculated according to the following equation:

$$SA = (\frac{A_0 - A_1}{A_0}) \cdot 100$$

where A_0 is the absorbance of the blank and A_1 is the absorbance in the presence of the extract. Ascorbic acid was used as positive control.

2.11.2. ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) Assay

ABTS radical cation ($ABTS^+$) was produced by the reaction of a 7 mM ABTS solution with 2.45 mM potassium persulphate [22]. The mixture was stored in the dark at room temperature for 12 h before use. The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. The ABTS scavenging ability (SA) was calculated according to the equation:

$$SA = (\frac{A_0 - A}{A_0}) \cdot 100$$

where A_0 is the absorbance of the control reaction and A is the absorbance in the presence of sample. Ascorbic acid was used as positive control.

2.11.3. FRAP Assay

The FRAP test is based on the redox reaction that involves TPTZ (2,4,6-tripyridyl-*s*-triazine)-Fe^{3+} complex. The absorbance of the reaction mixture was measured at 595 nm [22]. Ethanol solutions of known Fe (II) concentration, in the range of 50–500 µM ($FeSO_4$), were used for obtaining the calibration curve. The FRAP value represents the *ratio* between the slope of the linear plot for reducing Fe^{3+}-TPTZ reagent by different samples compared to the slope of the plot for $FeSO_4$. BHT was used as positive control.

2.11.4. β-Carotene Bleaching Test

In β-carotene bleaching test a mixture of linoleic acid (0.02 mL), Tween 20 (0.2 mL), and β-carotene (1 mL; concentration of 0.2 mg/mL) was prepared [22]. After evaporation of solvent and dilution with water, the emulsion (5 mL) was added into tubes containing clarified pomegranate juice. The tubes were placed at 45 °C in a water bath for 60 min. The absorbance measurement was carried out at 470 nm at initial time (t = 0) and after 60 min of incubation. The antioxidant activity (AA) was measured by using the following equation:

$$AA = \left[1 - \frac{(A_0 - A_t)}{(A^{\circ}_0 - A^{\circ}_t)}\right] \cdot 100$$

where A_0 and $A^{\circ}{}_0$ are the absorbance values measured at the initial incubation time for samples/standard and control, respectively; A_t and $A^{\circ}{}_t$ are the absorbance values for samples/standard and control respectively at t = 60 min. Propyl gallate was used as positive control.

2.12. Carbohydrate-Hydrolysing Enzymes Inhibitory Activity

2.12.1. α-Amylase Inhibitory Activity

The α-amylase inhibition assay was performed as previously described [23]. The α-amylase solution was prepared by adding 0.0253 g of enzyme in 100 mL of cold distilled water. The starch solution was prepared by stirring 0.125 g of potato starch in 25 mL of sodium phosphate buffer 20 mM and sodium chloride 6.7 mM (65 °C for 15 min). The colorimetric reagent was prepared mixing a sodium potassium tartrate solution and 96 mM 3,5-dinitrosalicylic acid solution. Clarified juice and control were added to the starch solution and left to react with α-amylase solution at 25 °C for 5 min. Acarbose was used as positive control. The enzyme inhibition (I) was calculated by using spectrophotometric data (at 540 nm) and by following equation:

$$\%I = 100 - \left(\frac{[maltose]\,test}{[maltose]\,control}\right) \cdot 100$$

2.12.2. α-Glucosidase Inhibition Assay

In α-glucosidase inhibition assay, a maltose solution was prepared by dissolving 12 g of maltose in 300 mL of 50 mM sodium acetate buffer [23]. The enzyme solution was prepared by adding 1 mg of enzyme (10 units/mg) in 10 mL of ice-cold distilled water. DIAN solution was prepared by dissolving 1 tablet in 25 mL of distilled water. PGO system-color reagent solution was obtained by dissolving 1 capsule in 100 mL of ice-cold distilled water. Both clarified juice and control were added to maltose solution and left equilibrate for 5 min at 37 °C. The addition of α-glucosidase solution started the reaction. After 30 min of incubation at 37 °C, the reaction was stopped by adding perchloric acid solution. The supernatant of tube of step one was mixed with DIAN and PGO and was left to incubate

at 37 °C for 30 min. Acarbose was used as positive control. The enzyme inhibition was calculated by using spectrophotometric data (at 500 nm) and by following equation:

$$\%I = 100 - (\frac{[\text{glucose}]\,test}{[\text{glucose}]\,control}) \cdot 100$$

2.13. Rejection

The rejection (R) of UF membranes towards specific compounds was calculated as follows:

$$R = (1 - \frac{C_p}{C_f}) \cdot 100$$

where C_p and C_f are the concentration of a specific component in the permeate and feed, respectively.

2.14. Statistical Analysis

Results were expressed as means ± standard deviation (SD) (n = 3). Prism Graphpad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to calculate the inhibitory concentration 50% (IC_{50}). Differences were evaluated by two-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test (** $p < 0.01$; α= 0.05). The concentration-response curve was obtained by plotting the percentage of inhibition versus the concentrations. *Pearson's* correlation coefficient was calculated using Microsoft Excel 2003.

3. Results and Discussion

3.1. Membrane Characterization

Figure 1 shows the SEM images of cross-sections of PVDF and PSU HF. In particular, PVDF membranes were made up mainly of finger-like macrovoids crossing the entire section of the fiber between two sponge-like surfaces. Indeed, as reported by Sukitpaneenit and Chung [24], when water is used as a coagulation bath, a finger-like structure is the predominant morphology that can be encountered in PVDF HF. This can be mostly attributed to the fast liquid-liquid de-mixing occurring during the coagulation of the fibers. Moreover, the presence of the pore former, PVP K17, can interfere with the de-mixing rate of the solution, leading to the formation of finger-like structures and macrovoids [25].

Regarding the bore fluid effect, it is well known in literature that the co-presence of solvents and alcohols slows down the de-mixing rate leading to a reduction of the finger-like structure [24,26]. However, due to the particular dope solution and spinning temperature employed, the kinetic enhancement of de-mixing prevailed on the inner coagulant effect. For this reason, PVDF HF with a finger-like structure were obtained in this work.

In PSU fibers, on the contrary, the finger-like macrovoids were mainly limited to the inner side and central portion of the fiber, while they were replaced by a sponge-like structure at the outer surface of the fiber. This aspect can be justified considering that the coagulation of the inner layer of the fibers starts immediately after the extrusion due to the contact with the bore fluid, while the outer layer passes through an air gap of 25.5 cm before immersion in the coagulation bath. The delayed coagulation of the outer layer, therefore, can lead to the fading of the macrovoids in favor of a sponge-like structure [26].

Lee et al. [27] studied the effect of PVP concentration on the morphology of PSU membranes prepared by NIPS. At small concentrations of PVP (2.5 wt%–7.5 wt%) in the casting solution, the formation of large macrovoids and finger structures was observed and it was governed by thermodynamic and kinetic properties. However, further increments of PVP concentration (up to 20 wt%) led to the suppression of macrovoids since the rheological hindrance, caused by an increase in dope solution viscosity, was prevailing. Therefore, the asymmetric structure of PSU fibers obtained

can be the result of a combined effect between rheological properties, thermodynamic enhancement, and kinetic hindrance.

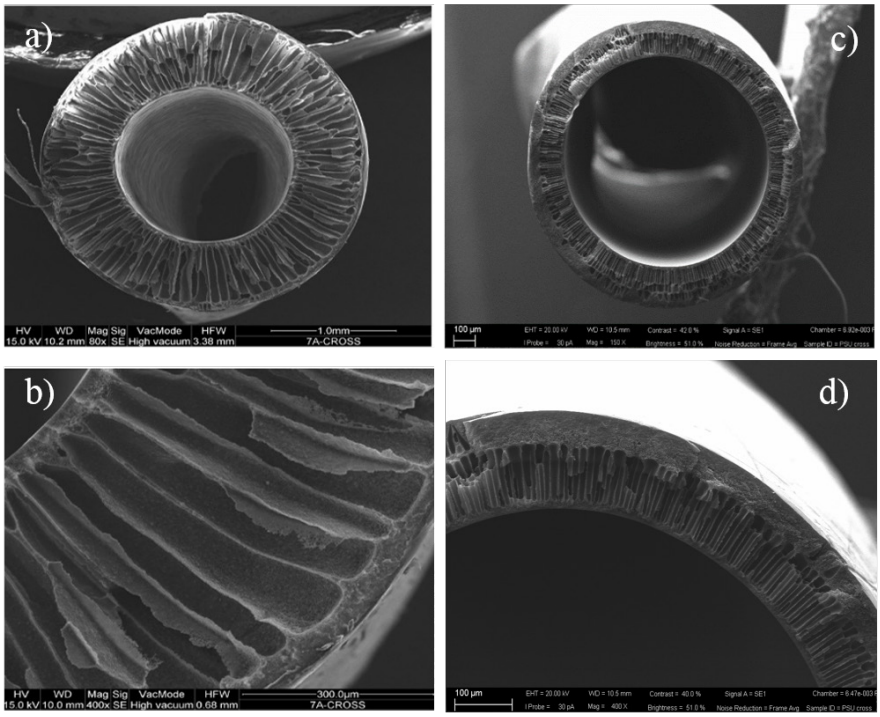

Figure 1. Scanning electron microscopy (SEM) images of the cross-section of polyvinylidene fluoride (PVDF) (**a**) and polysulfone (PSU) (**c**) hollow fiber (HF) membranes with their respective magnifications (**b**,**d**).

The main properties exhibited by the HF fibers produced are summarized in Table 2. In both cases PVDF and PSU fibers presented similar values of bubble point (1.1 bar) and same average pore size (0.13 μm). The average pore size places both membranes in the range of open ultrafiltration.

Table 2. Main properties of PVDF and PSU HF.

Parameter	Fiber	
	PVDF	**PSU**
O.D. (mm)	2.49 ± 0.44	1.30 ± 0.41
I.D. (mm)	1.44 ± 0.56	0.94 ± 0.32
Thickness (mm)	0.53 ± 0.8	0.18 ± 0.54
Emod (N/mm^2)	40 ± 2.5	126.42 ± 3.19
εbreak (%)	79.5 ± 3.3	42.91 ± 6.30
Bubble point (bar)	1.10	1.175
Pore diameter (μm)	0.13	0.13
Porosity (%)	88 ± 0.51	78 ± 0.22
Contact angle (°)	72 ± 3	63 ± 3
Water permeability (L/m^2 h bar)	343 ± 40	139 ± 23

Abbreviations: Emod: Young's modulus; εbreak: elongation at break.

PVDF fibers resulted in higher porosity (88%) in comparison to PSU ones (78%). The higher porosity of PVDF HF is, therefore, related to their morphology characterised by a large amount of macrovoids in comparison to PSU ones, which reduces its mechanical resistance [28]. PVDF membranes, in fact, presented a lower mechanical resistance and higher elasticity in comparison to PSU ones as evidenced by the Young's modulus values and elongation, respectively.

Both membranes exhibited a hydrophilic nature that was more pronounced for PSU HF (presenting a water contact angle of 63 ± 3°) than PVDF ones (72 ± 3°).

The enhancement in membrane hydrophilicity could be related to the presence of the hydrophilic PVP into the polymeric matrix and not washed away during membrane post-treatment with water [29–31]. The water permeability is reported in Table 2.

The permeability was found to be higher—more than double—for PVDF HF (343 ± 40 L/m^2 h bar) in comparison to PSU ones (139 ± 23 L/m^2 h bar). PVDF HF membranes, in fact, were characterized by a prevalence of finger-like structure and higher porosity, which were able to enhance the water permeation (despite their higher thickness) through the membrane.

3.2. Pomegranate Juice Filtration

In Figure 2 the steady-state permeate flux as a function of TMP for both prepared membranes is shown. According to the gel polarization model, a limiting flux is reached at a TMP value of 0.6 bar due to the concentration polarization phenomenon that arises as the feed solution is convected towards the membrane, where the separation of suspended and soluble solids from bulk solution takes place. The formation of a viscous and gelatinous-type layer is responsible for an additional resistance to the permeate flux in addition to that of the membrane [32]. PVDF HF membranes showed higher initial permeate fluxes (8.5 L/m^2 h) in comparison to PSU membranes (7.5 L/m^2 h) when the juice was clarified according to a batch concentration configuration. For both membranes a decrease of permeate flux by increasing the volume reduction factor was observed due to concentration polarization and fouling phenomena until reaching a steady-state value of about 3 L/m^2 h.

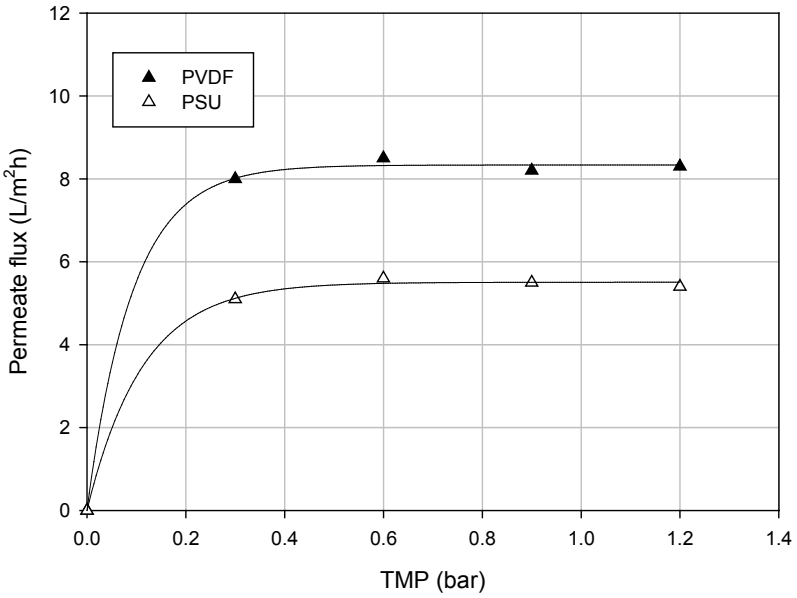

Figure 2. Permeate flux as a function of transmembrane pressures (TMP) for PVDF and PSU HF membranes.

The formation of a cake layer was found to be the main fouling mechanism occurring during the clarification of pomegranate juice with flat-sheet PVDF membranes having pore sizes of 0.22 and 0.45 µm [16]. Other fouling mechanisms, such as blocking, standard blocking, and intermediate blocking were less observed because they were probably hindered by the high thickness of the formed cake layer.

A flux of about 3 L/m^2 hwas obtained for PVDF membranes with a pore size of 0.22 µm after 30 min of filtration. The initial water permeability of PVDF membrane dropped about 99% after

filtration with pomegranate juice, while the decrease for PSU HF was about 95%. Following water flushing and enzymatic cleaning, the initial water permeability was totally restored as the initial value.

3.3. Phytochemicals Content

The health benefits of fruits such as pomegranate are mainly attributed to the presence of phenols, vitamins, and carotenoids. Increased intake of phenols, as demonstrated by different epidemiological studies, was associated with a reduced risk of diseases such as cardiovascular and neurodegenerative diseases, and cancer [33,34].

The beneficial effects of polyphenols are mainly ascribed to their capacity to counteract conditions of oxidative stress that accompany these pathologies. Several polyphenols have been demonstrated to have clear antioxidant properties in vitro as they can act as chain breakers or radicals scavengers depending on their chemical structures, which also influence their antioxidant power [35]. A hierarchy has been established for the different polyphenolic compounds within each class on the basis of their capability to protect lipids, proteins, or DNA against oxidative injury [36].

Table 3 reports the chemical and physical properties of fresh and clarified pomegranate juice by using both types of HF. The clarification process by HF was able to totally retain the suspended solids concentrating them in the retentate and allowing the production of a clarified juice.

Table 3. Composition of pomegranate juice clarified by PVDF and PSU membranes.

Parameter	Initial Juice	PVDF-P	PSU-P
pH	3.99 ± 0.08	3.64 ± 0.07	4.06 ± 0.08
Suspended solids (% *w*/*w*)	4.16 ± 0.08	-	-
Total soluble solids (Brix)	22.1 ± 0.44	21.9 ± 0.43	21.4 ± 0.43
Total phenols [a]	1996.7 ± 38.9	1934.3 ± 41.9	1888.1 ± 22.7
Total flavonoids [b]	295.4 ± 3.6	285.0 ± 2.4	196.9 ± 3.9
Total anthocyanins [c]	124.7 ± 2.9	110.1 ± 2.3	64.5 ± 1.8
Ascorbic acid [d]	13.8 ± 0.5	8.6 ± 0.1	10.5 ± 0.3

PVDF-P: Permeate obtained by PVDF membrane; PSU-P: Permeate obtained by PSU membrane. Data are expressed as media ± SD (standard deviation) ($n = 3$). [a] mg of chlorogrnic acid equivalents /L. [b] mg of quercetin equivalents /L. [c] mg cianidin-3-glucoside/L. [d] mg/100 mL.

The variation in soluble solids was not significant between the fresh and the clarified juice due to their permeation through the membrane. Even their pH was not significantly different. However, the retention of total phenols, total flavonoids, and total anthocyanins for both HF membranes presented some distinctions. Indeed, PVDF membranes presented a lower retention towards these compounds in comparison to PSU membranes (Figure 3). A similar trend was also observed by Galanakis et al. [37] in the ultrafiltration of winery sludge by using PSU and composite fluoropolymer (PVDF + hydrophilic cellulosic polymer) membranes. The composite fluoropolymer membrane presented, in fact, a lower retention towards phenols, flavonoids, and anthocyanins despite its lower molecular weight cut-off (1 kDa) in comparison to PSU membranes (100 and 20 kDa).

An explanation can lie in the different chemical nature of both polymers. PSU is made of a carbon chain alternating aromatic and aliphatic units, responsible of the hydrophobic character of the polymer, and oxygen and sulfur dioxide subunits providing the hydrophilic character of the polymer. PVDF, on the contrary, is made of alternating units of CH_2 and CF_2, conferring a hydrophobic nature to the material. The hydrophilic subunits of PSU polymer could be, therefore, more prone to create hydrogen bonds and Van-der-Waals interactions with the hydroxyl groups exhibited by polyphenols, flavonoids and anthocyanins with consequent adsorption of these components at membrane surface with the formation of fouling. PVDF HF, on the contrary, were less susceptible to hydrogen bonds and Van-der-Waals interactions making them more resistant to fouling and highly permeable to the considered compounds. These results confirm that the membrane material has a significant bearing on membrane-solute interactions, rather than the membrane pore size (which is the same for both

investigated membranes), highlighting its key contribution in the formation of fouling layers. The retention of PVDF membranes towards the anthocyanin content was similar to that observed by Mirsaeedghazi et al. [38] with PVDF membranes having a pore size of 0.22 and 0.45 μm.

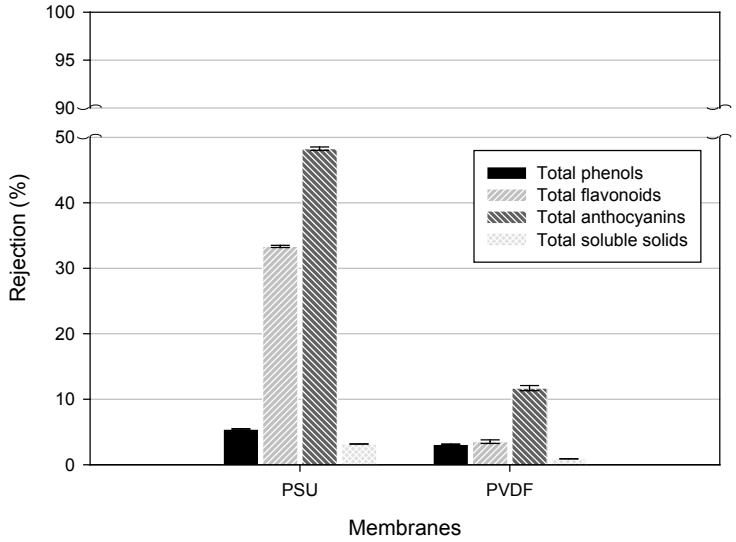

Figure 3. Rejection of PSU and PVDF HF membranes towards specific compounds of pomegranate juice.

3.4. Bioactivity of the Treated Juice

In this study, we have investigated the antioxidant effects of pomegranate juice clarified by PVDF and PSU membranes (PVDF-P and PSU-P, respectively) using different in vitro assays.

Radical scavenging activity was evaluated by two assays, namely ABTS and DPPH tests. Clarified pomegranate juice scavenged both radicals in a concentration-dependent manner. Results are reported in Table 4.

Table 4. Antioxidant activity of samples of pomegranate juice clarified by PVDF and PSU membranes.

Sample		DPPH Test (IC_{50} μg/mL)	ABTS Test (IC_{50} μg/mL)	FRAP Test (μM Fe(II)/g) [a]	β-Carotene Bleaching Test (IC_{50} μg/mL)	
					30 min	60 min
Clarified Juice	PVDF-P	733.08 ± 2.8	300.86 ± 2.7	13.83 ± 0.6	20.79 ± 1.4	43.22 ± 1.0
	PSU-P	42.41% [#]	467.80 ± 3.9	6.56 ± 0.4	69.03 ± 1.7	63.05 ± 2.6
Positive Control	Ascorbic acid	5.0 ± 0.8	1.0 ± 0.03			
	BHT			63.2 ± 4.5		
	Propyl gallate				1.0 ± 0.04	1.0 ± 0.03

PVDF-P: Permeate obtained by PVDF membrane; PSU-P: Permeate obtained by PSU membrane. Data are expressed as media ± SD (standard deviation) (n = 3). Positive control: ascorbic acid, BHT and propyl gallate. [a] Samples tested at the concentration 2.5 mg/mL. [#] at 1000 μg/mL. DPPH: One-way ANOVA *** $p < 0.0001$ (F = 214,700, R^2 = 1.0) followed by a multicomparison Dunnett's test: $\alpha = 0.05$; $p < 0.01$ compared with ascorbic acid. ABTS: One-way ANOVA *** $p < 0.0001$ (F = 22,780, R^2 = 0.999) followed by a multicomparison Dunnett's test: $\alpha = 0.05$; $p < 0.01$ compared with ascorbic acid. FRAP: One-way ANOVA *** $p < 0.0001$ (F = 611.8, R^2 = 0.996) followed by a multicomparison Dunnett's test: $\alpha = 0.05$; $p < 0.01$ compared with BHT. β-Carotene bleaching test 30 min incubation: One-way ANOVA *** $p < 0.0001$ (F = 1795, R^2 = 0.998) followed by a multicomparison Dunnett's test: $\alpha = 0.05$; $p < 0.01$ compared with propyl gallate. β-Carotene bleaching test 60 min incubation: One-way ANOVA *** $p < 0.0001$ (F = 1643, R^2 = 0.998) followed by a multicomparison Dunnett's test: $\alpha = 0.05$; $p < 0.01$ compared with propyl gallate.

In both assays, the use of PVDF membranes resulted in better radical scavenging activity. In particular, in ABTS test the IC_{50} value of PVDF-P was 1.5-time lower than PSU-P (300.86 μg/mL vs. 467.80 μg/mL, respectively). A similar trend was also observed in DPPH assay in which PVDF-P exhibited an IC_{50} value of 733.08 μg/mL while PSU-P showed a percentage of inhibition of 42.41% at 1000 μg/mL.

Total phenols, flavonoids, and anthocyanins are positively correlated with DPPH results ($r = 1$). A recent study, related to the effect of ultrasound waves on the efficiency of membrane clarification of pomegranate juice, showed a positive correlation between anthocyanins and DPPH radical scavenging activity of juice [39]. For the ABTS assay, correlation analysis revealed an *r*-value of 1 only for ascorbic acid content.

Since lipid peroxidation in the body is primarily the oxidative damage of cell membrane, it is also necessary to examine the effect of treated pomegranate juice on the lipid peroxidation [40]. In the β-carotene bleaching test, the oxidation of linoleic acid generates peroxyl free radicals due to the abstraction of a hydrogen atom from diallylic methylene groups of linoleic acid. The presence of phytochemical with antioxidant potential can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in this model. Clarification of pomegranate juice with PVDF membranes determined the highest protection on lipid peroxidation with IC_{50} values of 20.79 and 43.22 μg/mL after 30 and 60 min of incubation, respectively, with an increased antioxidant potency of 3.32 and 1.46, respectively, in comparison to the juice clarified by PSU membranes. A low ferric reducing ability was found for both PVDF and PSU membranes (13.83 and 6.56 μM Fe(II)/g, respectively). A *Pearson's* index ($r = 1$) was found for total phenols, flavonoids and anthocyanins content.

The effect of clarification process by using different approaches on juice health potential is controversial. In fact, Mirsaeedghazi et al. [38] reported a negative effect of PVDF membranes in the clarification of pomegranate juice in terms of reduction of total phenol content and, consequently, also of its antioxidant activity. More recently, Valero et al. [41] confirmed that clarification processes reduced the total phenol content in pomegranate juice despite having a positive effect on the antioxidant capacity. The interaction of phenolic compounds with other components in fruit juices has not been well investigated. In spite of this, synergistic and antagonistic interactions of two or more antioxidants have been documented in biological and model systems [42]. The higher antioxidant potential of clarified pomegranate juice could be explained through the removal of the antagonism between antioxidants and other juice constituents.

In a previous work, modified poly(ether ether ketone) HF membranes, with a dextran 68,800 MW rejection of 10%, were used to clarify pomegranate juice before a concentration step performed by osmotic distillation [43]. These membranes showed a rejection towards phenols and total antioxidant activity evaluated by ABTS test of 16.5% and 17.8%, respectively. Several works evidenced that the antioxidant activity of pomegranate juice may be related primarily to anthocyanin content, especially the 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin and secondly to tannins along with gallagyl-type tannins, ellagic acid derivatives, and other hydrolysable tannins [39,44].

Recent studies demonstrated that the flower, peel, and seeds of pomegranate exhibit hypoglycaemic activities in several in vitro and in vivo models [45–47]. Phenolic compounds also affect digestive enzymes involved in the hydrolysis of dietary carbohydrates. The inhibition of α-amylase and α-glucosidase by polyphenolic-rich plant extracts or isolated phenolic compounds may offer a natural dietary approach to preventing type 2 diabetes. Since pomegranate contains a high content of phytochemicals, the present study evaluates and compares the effects of permeates of both HF membranes on porcine pancreatic α-amylase and rat intestinal α-glucosidase enzyme activities in vitro. All analyzed samples inhibited carbohydrate-hydrolyzing enzymes in a concentration-dependent manner. Results are reported in Table 5.

The treatment with PVDF membranes produced a clarified juice with 2.9-time fold higher α-amylase inhibitory activity in comparison to PSU-P (IC_{50} value of 75.86 vs. 221.31 μg/mL, respectively). The same trend was observed also in α-glucosidase inhibition test with IC_{50} value of 69.07 μg/mL vs. 89.71 μg/mL for PVDF-P and PSU-P, respectively. PVDF-P showed an IC_{50} value lower than positive control acarbose (IC_{50} value 50.0 μg/mL).

Traditionally, pomegranate juice has been consumed for the treatment of diabetes mellitus [47]. There is some evidence that polyphenols from fruits and fruit products can also inhibit digestive

enzymes. McDougall et al. [48] reviewed that berry anthocyanins, ellagitannins, and proanthocyanidins inhibit α-glucosidase and α-amylase.

Table 5. Inhibitory α-amylase and α-glucosidase activity by samples of pomegranate juice clarified by PVDF and PSU membranes.

Sample		IC_{50} (μg/mL)	
		α-Amylase	**α-Glucosidase**
Clarified Juice	PVDF-P	75.86 ± 3.9 [a]	69.07 ± 2.1 [a]
	PSU-P	221.31 ± 4.9 [a]	89.71 ± 2.5 [a]
Positive Control	Acarbose	50.0 ± 0.9	35.5 ± 1.2

PVDF-P: Permeate obtained by PVDF membrane; PSU-P: Permeate obtained by PSU membrane. Data are expressed as media ± SD (n = 3). Acarbose was used as positive control. α-Amylase: One-way ANOVA *** $p < 0.0001$ followed by a multicomparison Dunnett's test: $\alpha = 0.05$ ($F = 6266$, $R^2 = 0.999$). α-Glucosidase: One-way ANOVA *** $p < 0.0001$ followed by a multicomparison Dunnett's test: $\alpha = 0.05$ ($F = 441.5$, $R^2 = 0.993$); [a] $p < 0.01$ compared with acarbose.

More recently, the ability of pomegranate tannins to inhibit α-amylase enzyme was demonstrated [49]. In general, larger and more complex tannins, such as those found in pomegranate juice, effectively inhibited the enzymes with various degrees of potency and in different sites on the enzyme. Pomegranate juice phenol-rich extract strongly inhibited rat intestinal α-glucosidase with an IC_{50} value of 922.8 μM of ellagic acid equivalent, whereas it was a weak inhibitor of porcine α-amylase (42% at maximum concentration tested). Punicalagin, punicalin, and ellagic acid, that characterized the ellagitannins-enriched fraction, were recognized as α-glucosidase inhibitors with IC_{50} values of 140.2, 191.4, and 380.9 μM, respectively. Kinetic analysis suggested that both pomegranate extract and ellagitannins inhibited α-glucosidase activity in a mixed mode. These effects are retained also by miming the gastro-intestinal digestion in vitro. In particular, during digestion punicalin and punicalagin concentration decreased. Despite this loss, the pomegranate extract retained a high inhibitory activity with a decrease in the amount of released glucose at the end of the gastro-intestinal digestion of 18% and 44% when the digestion was carried out with 2.35 or 4.7 mmol/L of total phenols, respectively [50].

Kam et al. [45] investigated the inhibitory activity of pomegranate flower, peel, seed and dried juice on α-amylase and α-glucosidase. The methanol flower extract inhibited both enzymes, while the methanol peel extract selectively inhibited the α-glucosidase. After water-ethyl acetate partition of flower extract, the ethyl acetate fraction showed the highest inhibitory activity (IC_{50} values of 200 and 98.7 μg/mL for α-amylase and α-glucosidase, respectively). In disagreement with our results, dried juice evidenced an inhibition less than 20% at 1000 μg/mL. The in vivo hypoglycaemic effect of pomegranate juice is largely unknown. Some researchers speculated that pomegranate might prevent diabetic sequelae via peroxisome proliferator-activated receptor-gamma binding and nitric oxide production [46]. A clinical study that involved 85 participants with type 2 diabetes demonstrated that 3 hours after fresh pomegranate juice administration, the juice decreased fasting serum glucose (FSG) levels, increased β-cell function, and decreased insulin resistance [51].

4. Conclusions

Three considerations inspired the present work: (1) the nutritional quality of processed foods is of great interest to the consumer and food processing industry due to its affect on health; (2) consumers are increasingly more aware of the influence of food on their health; and (3) over the last decade, several studies indicated pomegranate fruit as potential agent for the prevention and the treatment of type 2 diabetes. So, in this study the in vitro antioxidant properties and hypoglycaemic activities of a pomegranate juice clarified by two different hollow fiber membranes, PVDF and PSU, were investigated. PVDF and PSU HF were prepared by using two different polymeric solutions and

specific spinning conditions through the phase inversion process. Both fibers presented high porosity, hydrophilic surface, and pore dimensions in the range of open ultrafiltration, making them suitable in the clarification process of pomegranate juice.

Interesting differences between the two types of membranes have been highlighted. PVDF membranes presented a lower retention towards healthy phytochemicals, including flavonoids and anthocyanins, in comparison to PSU membranes. Consequently, the PVDF clarified juice exhibited greater antioxidant effects than the juice clarified with PSU membranes. In addition, the treatment with PVDF membranes enriched the juice in α-amylase and α-glucosidase inhibitors. The obtained results showed, for the first time, that the clarification of pomegranate juice by using PVDF membranes could be a suitable, easily applicable, and fast method to produce a juice rich in health- beneficial compounds that are of interest for the production of functional foods and beverages.

Author Contributions: Francesco Galiano and Alberto Figoli conceived and performed the experiments of hollow fibers preparation and characterization; Carmela Conidi and Alfredo Cassano conceived and performed the experiments related to pomegranate juice filtration; Francesco Menichini, Marco Bonesi, Monica R. Loizzo and Rosa Tundis conceived and performed the phytochemical analyses and the in vitro biological assays.

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