Article

Not peer-reviewed version

Unlocking the Bioactive Potential of Pomegranate Peels: A Green Extraction Approach

Giorgio Grillo , Giorgio Capaldi , Kristina Radošević , Željko Jakopović , Ksenija Markov , Mladen Brncic , Lorenzo Gallina , Emanuela Calcio Gaudino , Giancarlo Cravotto *

Posted Date: 1 August 2023

doi: 10.20944/preprints202308.0064.v1

Keywords: Pomegranate Peels; Green Extraction; Food-Waste Valorization; Polyphenols; Antioxidant Activity; Flavonoids; Anthocyanins; Green Metrics; Energetic Evaluation; Biological Activity

Article

Unlocking the Bioactive Potential of Pomegranate Peels: A Green Extraction Approach

Giorgio Grillo [1], Giorgio Capaldi [1], Kristina Radošević [2], Željko Jakopović [3], Ksenija Markov [3], Mladen Brncic [4], Lorenzo Gallina [1] Emanuela Calcio Gaudino [1] and Giancarlo Cravotto [1,*]

[1] Department of Drug Science and Technology, University of Turin, Via P. Giuria 9, 10235 Turin, Italy; giorgio.grillo@unito.it (G.G.); giorgio.capaldi@unito.it (G.Ca.); Emanuela Calcio Gaudino (E.C.G.)

[2] Laboratory for Cell Cultures, Applications and Biotransformations, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottojeva ulica 6, 10000 Zagreb, Croatia; kristina.radosevic@pbf.unizg.hr (K.R.)

[3] Laboratory for General Microbiology and Food Microbiology, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottojeva ulica 6, 10000 Zagreb, Croatia; zjakopovic@pbf.hr (Z.J.) kmarkov2@pbf.hr (K.M.)

[4] Department of Food Engineering, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia; mbrncic@pbf.hr (M.B.)

* Correspondence: giancarlo.cravotto@unito.it; Tel.: 011.6707183

Abstract: Pomegranate (*Punica granatum* L.) is well known for its high content in bioactives including polyphenols, flavonoids and tannins, which have been shown to exhibit a wide range of biological activity such as antioxidant, antimicrobial and anticancer effects. It is worth noting that the majority of these molecules are found in the peels (PP), which are usually disposed of after processing, causing a significant amount of waste, amounting to more than 3.6 million t/y. This work investigates MW-assisted extraction (MAE) in water for the recovery of antioxidants from PP, including the optimization of temperature and extraction times. The total phenolics, anthocyanin, flavonoid and tannin contents of the recovered extracts have been determined, as have their antioxidant activities, which were found to be 356.35 mgGAE/gextr., 303.97 μgCy3G/gextr., 37.28 mgQE/gextr., 56.48 mgGAE/gextr. and 1.43 μmolTE/gextr., respectively (according to the adopted reference). All results have been compared with those obtained using a conventional protocol. In addition, the potential for water recycling by means of nanofiltration downstream in optimized MAE has been investigated, leading to overall water reuse of approx. 75%. Energy consumption (20.92W/mgGAE) and the common green metrics, RME, E-Factor and PMI (PME), have been considered in evaluating the proposed PP valorization strategy. Finally, the biological activity of the main products has been assessed. The antimicrobial properties of the PP extracts against three Gram-positive and three Gram-negative bacteria, and their antiproliferative activity towards human cancer cells have been tested. The *S. aureus* bacteria was the most susceptible to the PP extracts. All tested products displayed antiproliferative activity in HeLa cells when higher concentrations were tested, with D-PP/NF being the most effective. This result was also confirmed in a clonogenic analysis, which generally indicated the possible anti-cancer activity of pomegranate peel extracts obtained using this green approach.

Keywords: pomegranate peels; green extraction; food-waste valorization; polyphenols; antioxidant activity; flavonoids; anthocyanins; green metrics; energetic evaluation; biological activity

1. Introduction

According to the FAO, approximately one-third of the World's food production is lost or wasted over the various stages of the food-supply chain. The extent of this loss varies greatly depending on the region and type of food. For fruit and vegetables, losses throughout the entire supply chain can be as high as 50%. This issue stands firmly in the field of one of the United Nations' Sustainable Development Objectives (OSD), which aims to reduce food waste by approximately 50% by 2050. [1]

This ambitious goal can be pursued using the Circular Economy concept, identified as a key principle for sustainable innovation, with an emphasis on creating a "zero waste" society and economy that aim to reduce waste and promote the efficient use of resources. In particular,

 doi:10.20944/preprints202308.0064.v1

(food)waste valorization refers to the process of utilizing this waste as a resource to create new products, rather than simply discarding it. Thus, it is necessary to emphasize that food waste, besides its applications in the energy, agronomy and animal feed sectors, can be a valuable feedstock for the recovery of bioactive compounds, including antioxidants and dietary fiber. [2–5] Extracting these compounds from food waste not only provides a sustainable approach to waste management but also generates value-added products that can be utilized in various applications, including as functional foods, nutraceuticals and cosmetics. [6,7] Furthermore, the residues generated after food-waste extraction for the recovery of valuable compounds can be further converted into new products, such as fermentation substrates, [8] bioplastics, [9] fine chemicals [10] or used as a source of energy through aerobic digestion. [11,12]

However, the extraction and conversion processes must be optimized to ensure that high-quality bioactive compounds are recovered while green chemistry principles are fulfilled. The importance of green chemistry in influencing future industrial processes cannot be denied. However, a significant number of procedures that involve extractions and chemical processes still rely on conventional reactors and methods. [13] The fundamental principles of green extraction focus not only on minimizing waste, but also on optimizing process efficiency while mitigating, at the same time, risks to human health and the environment. [14] This approach to so-called "process intensification" can be mainly achieved using enabling technologies (such as microwaves, ultrasound, pulsed electric fields, supercritical fluid extraction, ohmic heating, etc.) that aim to maximize process heat and mass transfer, leading to increased yield/conversion rates and, thus, savings in time and energy. [15] In this context, water-based extractions play a pivotal role, with water being considered "green" by definition as it is an easily usable and non-hazardous solvent. [16] Water, with its high polarity, possesses the ability to dissolve a wide range of polar compounds. However, although certain molecules (*i.e.* polyphenols) exhibit limited polarity, using water under subcritical conditions is still a viable option. Significant physico-chemical changes occur under these conditions, and selecting the appropriate temperature can lead to the recovery of several classes of target compounds. [17]

Green metrics are key tools in assessing the environmental impact of chemical processes. They provide quantitative measures that evaluate the efficiency and sustainability of chemical reactions and processes. Reaction Mass Efficiency (RME), the E-factor and Process Mass Intensity (PMI) are three crucial and commonly used green metrics. [18]

RME is a metric that quantifies the use of reactants in a chemical reaction. It represents the proportion of the total mass of reactants that is converted into desired products (see Equation (1)).

$$Reaction\ Mass\ Efficecny\ (RME, \%) = \frac{Mass\ of\ Product}{Mass\ of\ Matrix} \cdot 100 \qquad (1)$$

Higher reaction mass efficiency indicates a more efficient use of raw materials and reduced waste generation. This parameter, typical in organic synthesis, can be adapted for extraction procedures (with lower values being expected, naturally). [19]

The E-factor, or environmental factor, is a measure of the waste generated during a process. It is calculated by dividing the total mass of waste generated by the mass of the desired product (see Equation (2)). A lower E-factor signifies a more sustainable process with minimal waste generation. [20]

$$E - Factor = \frac{Total\ Mass\ of\ Waste}{Mass\ of\ Product} \qquad (2)$$

Process mass intensity (PMI) is a metric that evaluates the efficiency of a chemical process by considering the overall mass of all materials, including reactants, solvents, catalysts and products, per unit of desired product (see Equation (3)). Lower process mass intensity indicates a more resource-efficient process, as this indicates reduced material usage and waste generation. Another way to express PMI is the Process Mass Efficiency (PME), which is expressed as a percentage and is directly proportional to the overall process sustainability (see Equation (4)). [21]

$$Process\ Mass\ Intensity\ (PMI) = \frac{Total\ Mass\ in\ the\ Process}{Mass\ of\ Product} \qquad (3)$$

 doi:10.20944/preprints202308.0064.v1

$$Process\ Mass\ Efficency\ (PME, \%) = \frac{1}{PMI} \cdot 100 \quad (4)$$

These green metrics play a crucial role in guiding sustainable-process design and optimization. Using green metrics, the chemical industry can strive for more sustainable practices, minimize waste, conserve resources and reduce the overall environmental footprint of chemical processes.

This present study, the investigation of a new sustainable approach for the valorization of Pomegranate peels (PP) as a food residue using microwave-assisted extraction (MAE) with water as the solvent medium, fits well into this context.

Pomegranate (*Punica granatum* L.) is one of the world's oldest known fruits, is deeply embedded in the cultures of the Mediterranean region and widely cultivated in many countries due to its numerous health benefits. [22] Its production is estimated to be about 8.1 million tons worldwide, with Turkey being the fourth-largest producer after India, China and Iran. [23] The fruit contains bioactive compounds such as polyphenols, flavonoids, and tannins, which have been shown to exhibit a range of biological activity including antioxidant, anticancer and anti-inflammatory effects. [24,25] However, the majority of these bioactive compounds are found in the peel and seeds, which are usually discarded during the processing of pomegranate fruit, leading to significant waste and environmental concerns. PP are generated during the processing of fruits, which involves the removal of the outer skin and the separation of the seeds and juice. The generated quantity depends on the processing method and the source of the fruit, but typically ranges from 30% to 50% of the total fruit weight. [26] We can therefore estimate, considering the abovementioned global production of the fruit, that around 3.6 million tons of waste are produced per year. This enormous amount of residue presents environmental and health risks, as it contributes to environmental pollution and disposal issues, particularly if not managed properly. Finding appropriate methods for the extraction of bioactive compounds from pomegranate residues and their transformation into added-value products is essential.

In recent years, there has been increasing interest in the recovery of bioactive compounds from PP using non-conventional technologies including MAE, ultrasound-assisted extraction (UAE), Supercritical Fluid Extraction (SFE) and Enzymatic assisted extraction (EAE), among others. [27] These innovative extraction technologies have shown great potential in improving the efficiency of the recovery of bioactives from biomass waste while reducing solvent usage and processing times. A recent review by Cano-Lamadrid *et al*., (2022) has focused on the valorization of pomegranate by-products and outlined several sustainable approaches to PP extraction, of which microwaves emerge as being particularly promising. [27] However, the reviewers concluded that: "although there are relevant and promising results, they are non-unanimous and scarce. Therefore, more research on MAE and comparison with other green techniques is required". Our present paper therefore aims to contribute to the development of more sustainable and efficient methods for the MW-assisted recovery of bioactives from PP, with potential applications in the food, pharmaceutical and nutraceutical industries. To further support the produced results, the optimized samples were also evaluated in terms of energy consumption and biological activity.

In particular, MW-assisted water extraction has been exploited for the recovery of bioactives from PP waste, with both temperatures and extraction times being optimized. The antioxidant activity of the MAE extracts has been evaluated in terms of DPPH· scavenging activity (2,2-diphenyl-1-picrylhydrazyl assay) and the results have been compared with those of the extracts obtained from the conventional protocol. Moreover, the total phenolic, anthocyanin, flavonoid and tannin contents of the recovered PP extracts have been determined. Finally, the biological activity of the PP extracts, in terms of antibacterial activity toward Gram-positive and Gram-negative bacteria, has been investigated as has its antiproliferative activity in a HeLa cell line.

In addition, in order ensure that the protocol is in line with the requisites of sustainable and eco-friendly protocols, a preliminary evaluation of the potential water recycling from the aqueous PP extracts, using membrane filtration via a nanofiltration protocol, has been performed on the optimized MAE. This downstream strategy may not only enable water reuse for further extraction cycles, but can also concentrate the extracted antioxidants, dramatically reducing process times and

 doi:10.20944/preprints202308.0064.v1

energy consumption. Moreover, the main green metrics, RME, E-Factor and PMI (PME), have been exploited to evaluate the proposed MAE approach for PP valorization.

2. Materials and Methods

2.1. Pomegranate Peels and Chemicals

The PP utilized in this study were sourced fresh from Tropical Food Machinery (Parma, Italy), stored under frozen conditions at -20 °C and protected from light. A series of tests were conducted on the fresh peels, while a separate fraction was subjected to drying at 45 °C for a duration of 12 hours in a ventilated laboratory oven (FALC Instruments, Treviglio, Italy). In order to determine the moisture content and the proportions of the organic and inorganic fractions, a thermogravimetric investigation was carried out using a muffle furnace (Nabertherm GmbH, Lilienthal, Germany). The experimental protocol involved dehydration at 100 °C for 12 hours, followed by calcination at 650 °C for 4 hours (see Table 1). All solvents and reagents utilized in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Thermogravimetric analysis of F-PP and D-PP.

Fraction	Percentage Composition (%)	
	Fresh Matrix	Dried Matrix
Water	75.52	3.50
Organic	23.86	94.06
Inorganic	0.62	2.44

2.2. MW-Assisted Extraction (MAE) of Pomegranate Peels in Water

The MAE experiments were conducted using an MW multimodal reactor (SynthWAVE, Milestone, Bergamo, Italy) equipped with external inert gas feeding capability (N_2). The optimization of the extraction protocol was performed using 1g of either fresh pomegranate peels (F-PP) or dried pomegranate peels (D-PP) with a solid-to-liquid ratio of 1:30. The screening explored three different extraction temperatures (100 °C, 125 °C and 150 °C) with a MW power of 1500 W and three different time intervals (10, 20 and 30 minutes). Agitation was provided by a magnetic stirrer working at *ca.* 650 rpm.

In order to minimize oxidative stress in the biomass, each test was preceded by three N_2 purges to eliminate atmospheric oxygen. Subsequently, the reaction chamber was pressurized with an appropriate amount of N_2 to prevent water ebullition (5–10 bars). Following the optimized protocol, a scaled-up experiment was conducted using 20 grams of biomass and 600 mL of solvent in a 1 L Teflon vessel, mixed using a mechanical stirrer at 650 rpm. The extraction process was performed at 100 °C for 10 minutes, utilizing both fresh and dried peels. Furthermore, the same conditions were employed for the extraction of D-PP, utilizing the permeate recycled from membrane nanofiltration.

After extraction, the resulting solutions were subjected to vacuum filtration after centrifugation at 4200 rpm to separate the solid particles. The dry extracts were subsequently recovered via freeze-drying (LyoQuest-85, Telstar, Madrid, Spain), weighed and stored at 4 °C for further analysis.

2.3. Hydroalcoholic Extraction of Pomegranate Peels

A conventional hydroalcoholic extraction was chosen as the benchmark protocol for pomegranate peels, for use as a comparison with the principal results achieved by means of MAE. The method involved reflux conditions using a hydroalcoholic solvent composition of 70% ethanol and 30% distilled water. 5g of dry peels were subjected to extraction with 150 mL of the hydroalcoholic solution, resulting in a solid-to-liquid ratio of 1:30. The extraction was carried out for 1 hour. Reflux conditions were achieved by employing an oil bath equipped with a magnetic stirrer (650 rpm). After extraction, the resulting solution was subjected to centrifugation at 4200 rpm, to separate the solid particles, and was then filtered under vacuum. The ethanolic fraction was removed

 doi:10.20944/preprints202308.0064.v1

using a rotavapor system operating at 40 °C and 175 mbar. The residual water fraction was removed under freeze-drying using a LyoQuest-85 freeze dryer (Telstar, Madrid, Spain). The dried extracts were weighed and stored at 4 °C for further analysis.

2.4. Membrane Nanofiltration (NF) for Bioactives Concentration and Water Recovery

Membrane nanofiltration (NF) was conducted on a 2 L solution of the D-PP extract that was produced under the optimized conditions (solid/liquid ratio of 1:30, 100 °C, 10 min). During this study, a pilot membrane filtration skid was used (PB100, Hydro Air Research Srl, Lodi, Italy). The system was equipped with a DKU 1812 NF membrane (filtering area: 0.38 m^2, molecular weight cut-off range: 150–300 Da). During the process, the retentate was recirculated within the system, while the permeate was continuously separated and collected, working under appropriate counter-pressure (5 bars). The separation was performed for approximately 25 minutes. As a result, 500 mL of retentate and 1500 mL of permeate, which were freeze-dried, weighed and stored at 4 °C for future analysis, were obtained from the 2 L solution. Part of the permeate was used "as obtained" for PP MAE, to evaluate its recyclability in the process.

2.5. Colorimetric Tests on Pomegranate Peel Extracts: Quali-Quantitative Characterization

2.5.1. Total Phenolic Content (TPC)

The determination of the Total Phenolic Content (TPC) followed a modified version of the *Folin–Ciocalteu* procedure described by Hillis and Swain. [28] For each extract, an aliquot of 0.25 mL was combined with 0.5 mL of a 10 % *w/v* Na_2CO_3 solution, followed by the addition of 0.25 mL of *Folin–Ciocalteu* reagent. The resulting solution was immediately diluted to a final volume of 5 mL using distilled water and thoroughly mixed. After allowing the solution to stand for 25 minutes, absorbance was measured at 725 nm using a Cary 60 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). A calibration curve was prepared using gallic acid solutions ranging from 0.01 to 0.45 mg/mL. TPC is expressed as mg/g of Gallic Acid Equivalents (GAE) over the extract (referred to as TPC selectivity) and over the matrix (referred as TPC Yield).

2.5.2. Total Anthocyanin Content (TAC)

The Total Anthocyanin Content (TAC) was determined using the pH differential method. [29] The dried extract was dissolved in deionized water to achieve a concentration of approximately 1.5 mg/mL. Two solutions were prepared by mixing 1 mL of the sample with 5 mL of potassium chloride buffer (0.025 M KCl, pH 1 with HCl) and sodium acetate buffer (0.4 M CH_3COONa, pH 4.5 with HCl). After allowing the solutions to equilibrate for 5 minutes, absorbance was measured at 510 nm and 700 nm using a Cary 60 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Deionized water was used as a blank. The resulting sample absorbance was calculated using Equations (5) and (6):

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5} \quad (5)$$

$$\text{Anthocyanin content (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1) \quad (6)$$

where MW is the molecular weight and ε is the molar absorptivity of the anthocyanin pigment in an acidic aqueous solvent. The values used in this formula correspond to Cyanidin-3-Glucoside (Cy3G, MW = 449.2 and ε = 26,900), meaning that the TAC results are expressed as Cy3G equivalents.

2.5.3. Total Tannin Content (TTC)

The determination of Total Tannin Content (TTC) was performed using the Peri and Pompei protocol [30] with some adjustments, as reported by Aimone *et al.* [31] In this analysis, 600 µL of hemisulfate cinchonine solution (0.5% w/v) was added to 600 µL of the extract solution in a 1.5 mL Eppendorf tube. The resulting mixture was shaken and then left overnight at 4 °C to promote the

 doi:10.20944/preprints202308.0064.v1

precipitation of tannate cinchonine. The sample was then centrifuged at 26,000 rpm for 2 min at 10 °C (Allegra 64R, Beckman Coulter Srl, Italy), and the supernatant, which contains a polyphenol-rich solution, was separated and analyzed using the *Folin–Ciocalteu* test (see Section 2.5.1). The total tannin content was calculated by determining the difference between the TPC of the fresh sample and the TPC obtained after tannin precipitation, and was expressed as Gallic Acid Equivalents (GAE).

2.5.4. Total Flavonoid Content (TFC)

The quantification of the Total Flavonoid Content (TFC) was performed using the colorimetric method described by Saikan Set *et al.*, with modifications. [32] A 0.5 mL aliquot of the sample (water solution) with a concentration of 1 mg/mL was mixed with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1M potassium acetate and 4.3 mL of 80% ethanol. The mixture was thoroughly mixed and allowed to stand at room temperature for 40 minutes. The absorbance of the supernatant was measured at 415 nm (Cary 60 UV–vis spectrophotometer, Agilent Technologies, Santa Clara, CA, USA) to determine the presence of flavonoids based on the development of a yellow color. The results were expressed as Quercetin equivalents in milligrams per gram (mgQE/g) of dry extract, using a standardized calibration curve.

2.5.5. Total Sugar Content (TSC)

The quantification of Total Carbohydrate Content (TSC) was conducted using the anthrone method, with modifications. [33] A calibration curve was prepared using a glucose-water solution, with dilutions ranging from 10 to 200 µg/mL, to serve as a reference for determining the sugar content in the samples. An aliquot of the sample solution, consisting of 1 mL with concentrations ranging from 0.01 to 0.5 mg/mL in deionized water, was mixed with 5 mL of anthrone reagent (0.2 g anthrone in 100 mL of concentrated 96% sulfuric acid). The solution was then heated at 100 °C for 20 minutes and absorbance was measured at 620 nm (Cary 60 UV–vis spectrophotometer, Agilent Technologies, Santa Clara, CA, USA). The increase in color intensity, from yellow to green/blue, was directly proportional to the carbohydrate concentration in the sample. The obtained data were analyzed using the glucose standard calibration curve and expressed as Glucose equivalents in milligrams per gram (mgGluE/g) of dry extract.

2.5.6. Antioxidant Activity: DPPH· Inhibition

The antioxidant activity of the extracts was assessed using the method described by Brand-Williams *et al.* [34] The antioxidant activity of the extracts was determined by measuring the inhibition of DPPH· radicals, which indicates their scavenging ability. The EC50 value, which represents the concentration of the extract required to inhibit 50% of the DPPH· radical, was determined as the parameter for scavenging activity. A Trolox methanolic solution served as the standard reference. The sample EC50 values were then compared to the EC50 values obtained from the standard to express the results as Trolox equivalents (µmolTE/mL). Various dry-extract concentrations were prepared via sequential dilution, and absorbance was measured at 515 nm using a Cary 60 UV-vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The obtained absorbance data were processed using Bobo Least Squares software with Probit regression analysis. [35] Blank samples containing only water and methanol were used for instrument zeroing, while blank samples with the dry extract, but without the DPPH· radical, were used to account for matrix effects. A reference sample containing water and the DPPH· radical was utilized to normalize the results and verify absorbance due to natural inhibition.

2.5.7. Cu-chelating Activity of PP Extracts

The chelating ability of the sample was evaluated using the pyrocatechol violet (PV) assay, following the method described by Santos *et al.* [36] PV forms a dark red complex with Cu^{2+} ions not bound by polyphenols in a slightly acidic medium, and the rate of color reduction indicates chelating activity. A solution containing the extract was mixed with sodium acetate buffer and a copper sulfate

 doi:10.20944/preprints202308.0064.v1

solution, and the PV solution was then added. After specific reaction times, absorbance was measured at 632 nm using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The inhibition of PV-Cu^{2+} complex formation was expressed as the percentage of inhibition compared to a reference solution prepared without the extract. Percentage inhibition was calculated using different sample concentrations, and the EC50 value was determined using probit regression and Bobo Least Squares software. [35] The sample EC50 values were then compared to the EC50 values obtained from an EDTA solution to express the results as EDTA equivalents (μmolEDTA/mL).

2.6. Biological Activity

2.6.1. Antibacterial Activity Test

Gram-negative bacterial strains of *Escherichia coli* 3014, *Pseudomonas aeruginosa* 3024, *Salmonella typhimurium* 3064 and Gram-positive bacterial strains of *Staphylococcus aureus* 3048, *Bacillus subtilits* 3045 and *Listeria monocytogenes* 3112 were obtained from the Collection of Microorganisms at the Laboratory of General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb (Zagreb, Croatia). All cultures were stored at -70 °C in nutrition broth (Biolife, Milano, Italy) with 30 % glycerol (by volume). To perform antibacterial activity test, microorganisms were firstly activated via incubation at 37 °C in the corresponding broths. The antibacterial activity of the PP extracts (F-PP, D-PP, D-PP/NF scaled-ups and hydroalcoholic benchmark) was tested on selected bacterial cultures in disc diffusion assays. Bacterial cultures were prepared and the CFUs of all the used suspensions were determined, and amounted to 10^7 – 10^8 CFU mL^{-1}. To perform the test, 100 μL of the bacterial suspensions was smeared, using a Drigalski rod, onto the nutrient agar plate and left to dry. After 30 minutes, sterile filter discs (6 mm) (Macherey-Nagel GmbH, Düren, Germany) were immersed into 50, 250 and 500 μg/mL of the PP extracts and placed on the surface of inoculated nutrient agar. After incubation, for 24 hours at 37 °C, inhibition was detected as clear halo diameter (mm). Discs with chloramphenicol (10 μg per disc) (Liofilchem, Roseto, Italy) were used as positive controls, while discs immersed in sterile water that was used to make the water solutions of the PP extracts acted as negative controls for all tested microorganisms. All assays were performed in triplicate.

2.6.2. Antiproliferation Assay on Human Cell Line

The antiproliferative activity of PP extracts (F-PP, D-PP, D-PP/NF scaled-ups and hydroalcoholic benchmark) was evaluated *in vitro* against an adherent human tumor cell line using the CellTiter AQueous One Solution Cell Proliferation Assay (Promega). HeLa (ATCC No. CCL-2) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen Corporation, U.K.) and maintained in BioLite Petri dishes (Thermo Fisher Scientific) in an incubator with a humidified atmosphere and 5% CO_2 at 37 °C. Experiments were performed three times with four parallels for each concentration of extract tested in BioLite 96-well plates (Thermo Fisher Scientific) that were seeded with exponentially growing cells at the indicated concentration (~3×10^4 cells per well in 100 μL of medium) and incubated for 24 h. Lyophilized extracts were dissolved in 10 mg/mL of water and further diluted in DMEM to the final tested concentration (50 μg/mL,100 μg/mL, 250 μg/mL, 500 μg/mL) before being applied to the cells. The control cells were untreated cells. After treatment, the cells were incubated for an additional 72 h, after which the CellTiter AQueous One Solution Cell Proliferation Assay was performed according to the manufacturer's instructions, with minor modifications. In brief, 10 μL of the CellTiter AQueous One Solution Cell Proliferation Reagent was added to each well and the cells were incubated for an additional 3 h, after which the absorbance was measured at 490 nm using a microplate reader (Tecan, Switzerland). Cell viability was expressed as the percentage of treated cells over control cells. Experiments were performed three times with four parallels for each concentration of tested compounds and data are expressed as mean ± S.D.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

2.6.3. Clonogenic Analysis

The clonogenic assays began by seeding pre-cultured HeLa cells in 6-well plates at an initial concentration of 200 cells in 2 mL of culture medium per well. The cells were incubated under optimal conditions and treated with the scaled-up extract of F-PP, D-PP, D-PP/NF and the hydroalcoholic benchmark at a concentration of 500 mg/mL after 24 h. Untreated control cells were also analyzed. Three days after the cells were treated, the growth medium containing the tested extracts was removed and replaced with fresh growth medium, after which, the plate with the HeLa cells was returned to the incubator for further cultivation. After treatment, the surviving cells required about 1–3 weeks to form colonies. In this work, the produced colonies were visible 19 days after the initial seeding of the cells. Staining the grown colonies with crystal-violet was begun by removing the growth medium and washing the cells with 1 mL of PBS buffer. 2.5 mL of methanol was then added to fix the cells, but was removed after 10 min. The plates were then allowed to air dry completely. A 0.5 % solution of crystal-violet was then added and incubated for 10 min. In the final step, the dye was removed and the colonies in the wells were rinsed with 1 mL of PBS buffer and deionized water. The number of colonies grown was then counted and the plating efficiency (PE) and survival fraction (SF) were calculated according to the equations in the protocol of Franken *et al.* [37] PE is the ratio of the number of colonies to the number of seeded cells, while SF is the number of colonies formed after the treatment of the cells, expressed as PE.

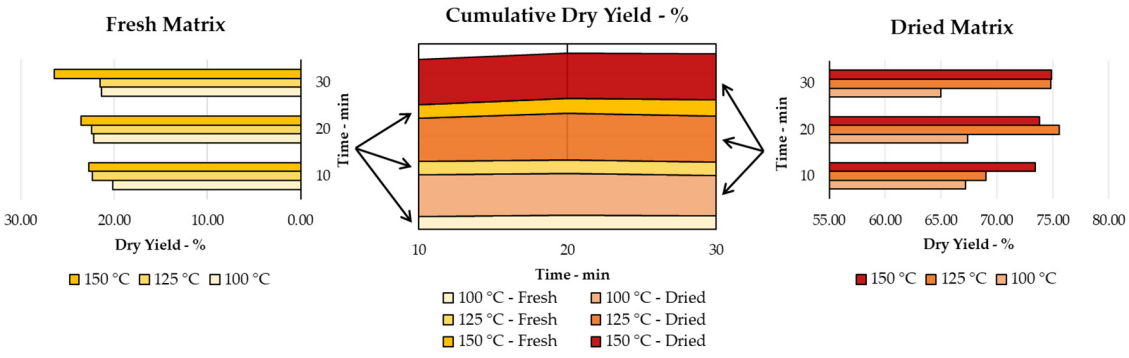
3. Results and Discussion

3.1. MW-Assisted Extraction of Pomegranate Peels (PP): Lab-Scale

The MAE of PP was screened using water as the solvent at three different temperatures (100 °C, 125 °C and 150 °C) for 10, 20 and 30 min. In order to explore the matrix effect, PP was extracted both in the fresh (F-PP) and in the dry form (D-PP). The products were characterized in terms of dry yield, TPC and TAC, and thus the best performing conditions were defined. The optimized protocol was then reproduced in a 1 L scale-up protocol, whose products were further characterized in terms of TFC, TTC, TSC, DPPH· inhibition and Cu-chelating activity (See Section 3.2). Biological activity was also assessed, with antibacterial activity against Gram-positive and Gram-negative bacterial strains, and antiproliferative activity toward a human tumor cell line being evaluated (See Section 3.3).

3.1.1. Dry Yield and Total Phenolic Content (TPC)

The dry yields of each extract from the MW-assisted protocols varied proportionally with temperature and process duration, for both F-PP and D-PP. Figure 1 reports the outcomes for each matrix together with the "Cumulative Dry Yield", where percentages are stacked to allow for direct comparisons. Thus, it worth noticing that D-PP (darker shades, larger areas) gave higher recoveries than F-PP (lighter shades, smaller areas) over all of the time and temperature conditions explored. Moreover, at fixed temperature, it is possible to observe how time increases do not strongly affect the process, as limited yield fluctuations were seen.



 doi:10.20944/preprints202308.0064.v1

Figure 1. Dry extraction yields for MAE of F-PP and D-PP: time and temperature screening; cumulative Dry Yield reports stacked percentage, for comparison.

In general, the best dried yields, for both F-PP (26.40%) and D-PP (74.85%), were achieved at 150 °C after 30 min of MAE, albeit with about 50% more extract being recovered from D-PP. However, comparable results can be achieved for the latter (approx. 74.8%) also at 125 °C after 30 minutes.

To better understand MAE-process trends, the *Folin-Ciocalteu* assay was adopted to evaluate the TPC of each recovered extract, in terms of selectivity and yield (see Section 2.5.1.). The first parameter, which expresses the amount of polyphenols (mgGAE) per gram of extract, is reported in Figure 2.

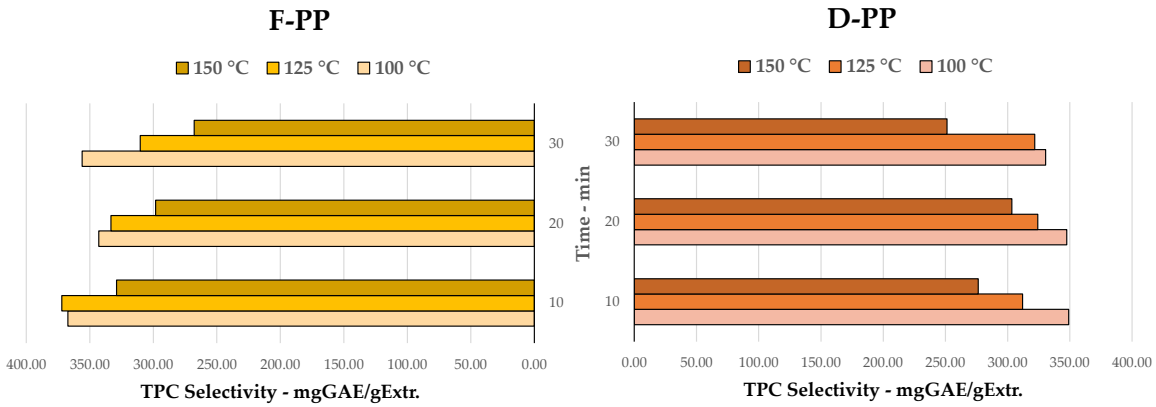


Figure 2. TPC selectivity for MAE of F-PP and D-PP, time and temperature screening.

TPC selectivity displayed the opposite trend to that of dry yield; polyphenol ratios decrease as the temperature and treatment time increase. This behavior, which was observed in both matrixes, may be related to a partial thermolability of the metabolites contained in PP, together with an increase in non-phenolic co-extracts. [38,39] The latter may be explained by the salts and oligo- or polysaccharides that are partially hydrolyzed, and thus solubilized, by the subcritical conditions, which become harsher the closer the process came to 150 °C. [40,41] This last hypothesis will be addressed further in the characterization performed on the optimized scaled-up samples (up to 600 mL, See Section 3.2.1). In detail, 367.36 and 349.14 mgGAE/gExtr were achieved at 100 °C after only 10 min, for F-PP and D-PP, respectively, whereas 267.90 and 251.30 mgGAE/gExtr were produced at 150 °C and 30 min. Unlike the dry extraction yield, the difference between the starting materials does not appear to be remarkable for TPC selectivity. In summary, TPC selectivity is favored when restricting MAE screenings to low temperatures and limited processing times.

In order to better understand how using either fresh or dried PPs can influence extraction-procedure effectiveness, we moved to an evaluation of process productivity. For this purpose, TPC yield was determined as the parameter of choice as it can represent a combination of polyphenol selectivity and dry extraction yield (mgGAE/gMatrix). The results are depicted in Figure 3A–C (100 °C, 125 °C and 150 °C, respectively).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

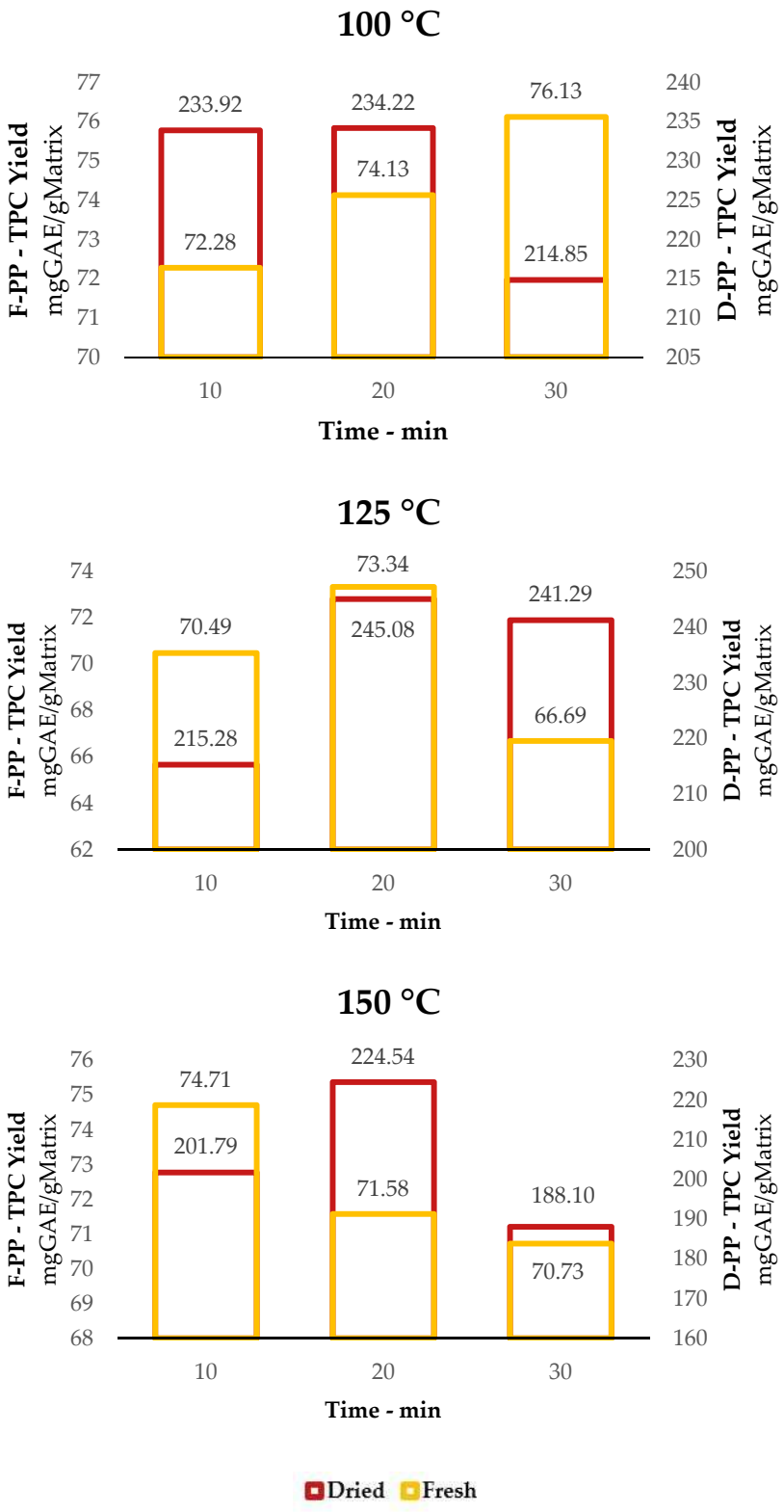


Figure 3. TPC yield for MAE of F-PP and D-PP at different temperatures: 100 °C, 125 °C and 150 °C.

As expected, the dramatic difference in the productivities of fresh and dried peels is clearly visible. The latter achieved up to 245.08 mgGAE/gMatrix, whereas 76.13 mgGAE/gMatrix was given by F-PP, representing a 3-fold increase (125 °C, 20 min and 100 °C, 30 min, respectively). Here the volumetric contraction caused by matrix dehydration is having a clear effect. Thus, for a given amount of treated biomass, the quantity of the processed "organic" fraction is clearly enhanced,

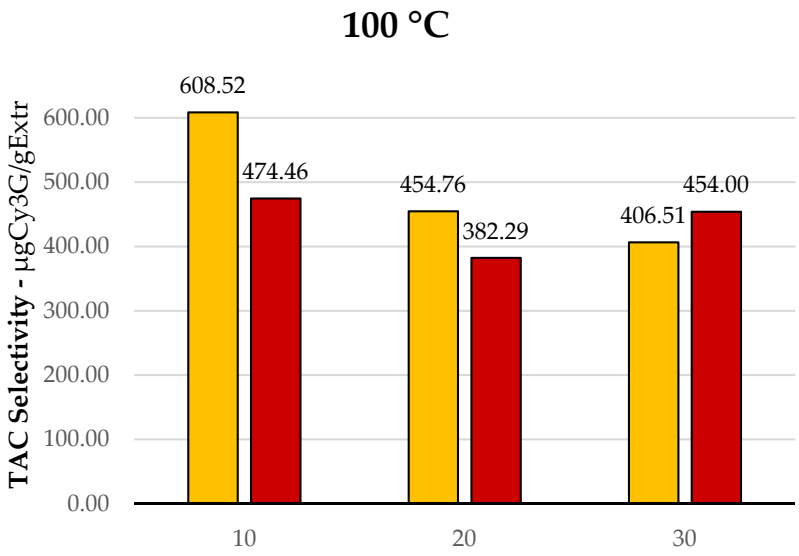
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

which has a clear positive effect on process productivity. The results of the TPC evaluations indicated that a general decrease in efficiency occurred in longer extractions (30 min), regardless of the process temperature, which seems to only slightly affect phenolic compounds. These data then appear to indicate that shorter and less harsh extraction conditions are to be preferred.

Once we had determined that D-PP provided the best productivity in terms of polyphenol recovery, this work was extended further in order to define whether the thermal dehydration step has an impact on two other aspects of the final product; anthocyanin content and overall energy consumption (Sections 3.1.2 and 3.2.2.).

3.1.2. Total Anthocyanin Content (TAC)

The first point in evaluating the effect of the drying process on PP valorization was to determine the recovery efficiency towards the most thermolabile class of metabolites present in PP; anthocyanins. The colorimetric assay adopted for this screening (See **Figure 4**) revealed, as expected, inverse proportionality to MAE extraction temperature, which worsened even further at longer times (from 10 to 30 min). Accordingly, anthocyanins are completely absent in the product achieved at 150 °C.



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

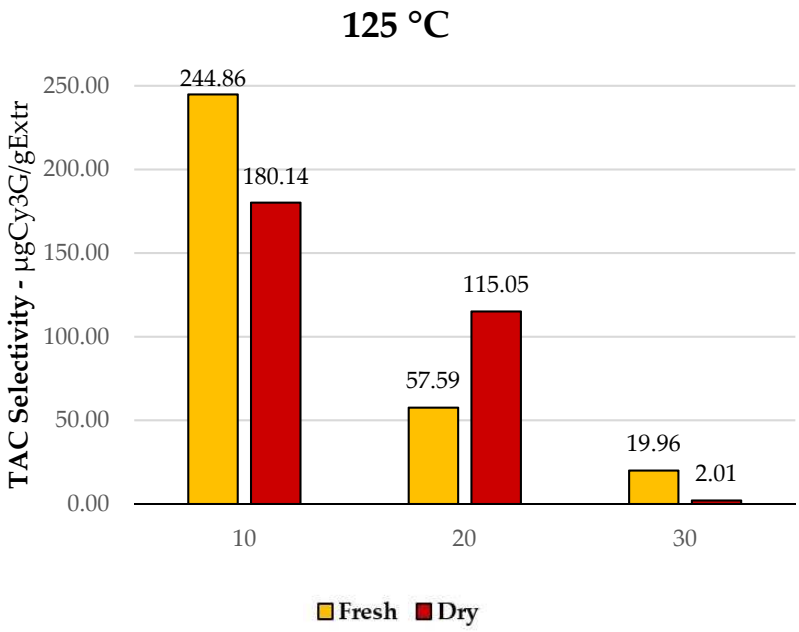


Figure 4. Extraction TAC for MAE of F-PP and D-PP, time and temperature dependence: 100 °C and 125 °C. 150 °C not reported due to absence of signal.

F-PP led to a maximum TAC of 608.52 µgCy3G/gExtr, whereas D-PP gave 474.46 µgCy3G/gExtr at 100 °C in 10 min of MAE, representing an approx. 1.5-fold increase in anthocyanin recovery. In general, it is possible to state that anthocyanins are not the main component available in the processed material, even in the fresh matrix. This can be extrapolated from the fact that, despite the high TAC selectivity, the overall TPC yield was significantly lower for F-PP (72.28 mg GAE/gMatrix *vs.* 608.52 µgCy3G/gExtr) than for D-PP (233.92 mg GAE/gMatrix *vs.* 474.46 µgCy3G/gExtr). Hence, from this point of view, the drying step does not seem to excessively affect the quality of the recovered extract. It worth noticing that this work is not strictly focused on anthocyanins, which, besides not being predominant in PP, tend to present highly fluctuating contents due to biomass handling and storing conditions. Moreover, these metabolites require particular extraction conditions (*i.e.* acidic media) that could potentially affect the sustainability of the whole valorization protocol and go beyond the approach proposed in this manuscript.

3.2. MW-Assisted Extraction of Pomegranate Peels (PP): Scale-Up

This work aims to design a valorization strategy for pomegranate residues by means of metabolite extraction in water under MAE. This ambitious task cannot ignore the need for a scaled-up approach because of the enormous amount of generated wastes. For this reason, the second part of this manuscript is dedicated to the exploitation of a 1 L MW-assisted extraction system, preliminary tackling this point. The process set-up was defined by merging the results of the lab-scale screenings, which indicate 100 °C and 10 min to be the best performing conditions for maximum bio-actives recovery. Both fresh and dried peels were tested for the sake of comparison.

3.2.1. Extract Characterization

Taking process intensification as the main focus, the extracts achieved by means of the MAE scale-up were characterized in TPC and TAC assays, together with additional TFC, TSC, TTC tests and an evaluation of preliminary antioxidant activity, in terms of radical-inhibition (DPPH·) and Cu-chelating capabilities (See Figure 5 and Table 2).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

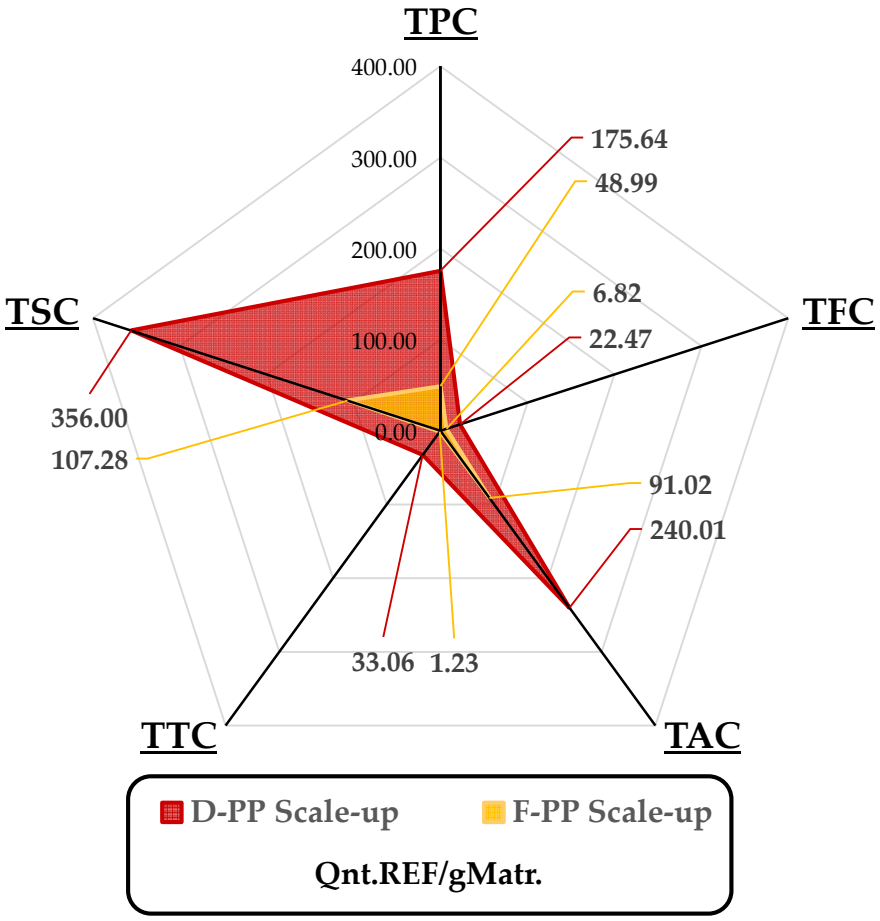


Figure 5. The main components of the quali-quantitative analysis for MAE scale-up for F-PP and D-PP. Yields are reported per gram of extracted matrix, reporting total weight of polyphenols, flavonoids, anthocyanins, tannins and sugars according to the adopted reference. In detail, TPC: mgGAE; TFC: mgQE; TAC: μgCy3G; TTC: mgGAE; TSC mgGLU.

The analyzed parameters are aligned with the trends observed in the lab-scale protocol, where metabolite selectivities do not substantially differ between F-PP and D-PP, whilst the corresponding yields, as depicted in Figure 5, are more than 3 times higher for the dried PP matrix, except for anthocyanin content.

Furthermore, a direct comparison of the lab-scale and scaled-up approaches (see Figure 6), makes it possible to evaluate the trend for the main analyzed components: TPC and TAC (first screenings in Sections 3.1.1 and 3.1.2, respectively). When a higher amount of matrix was processed, these two parameters registered a contraction in both selectivity and yield; approx. an average of 1.4-fold. It is worth noticing that a minimal performance drop can be assumed to be physiological when transposing a process towards higher volumes, due to the impossibility of achieving a completely "geometrical" scale-up.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

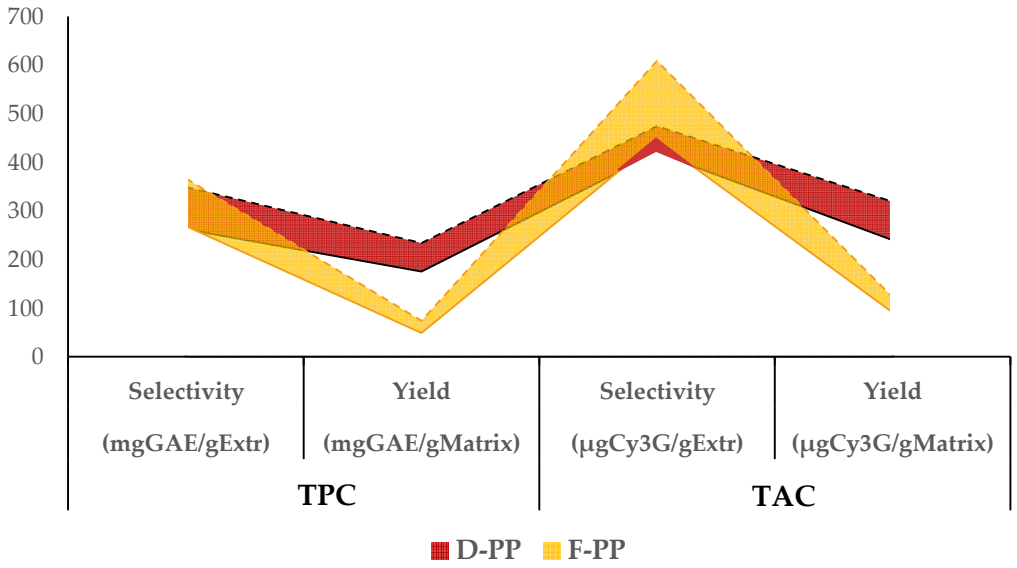


Figure 6. Comparison of TPC and TAC variation in scale-up transposition. Selectivity and yields are reported for D-PP and F-PP. Dashed lines: Lab-scale; Solid lines: Scaled-up.

As expected, greater variability in TAC can be observed in F-PP, due to its higher content in these metabolites. Similarly, this same matrix showed the smallest yield reductions (for both TPC and TAC), caused by the limited amount of extracted plant material, due to the high water content. Conversely, D-PP showed a more stable trend, with limited losses for all the explored parameters.

From this point onward, the scaled-up aqueous MAE protocol was explored and characterized further using both F-PP and D-PP, for the sake of comparison.

Polyphenolic compounds act as potent reactive oxygen and nitrogen species (ROS, RNS) scavengers, as well as metal chelating agents. Accordingly, these metabolites can be exploited for the prevention of mutation-related diseases due to their antioxidant features, [42] that act dynamically in the balance between direct ROS/RNS quenching and metal chelating capacity. [43,44]

The inhibition of DPPH· radicals and the Cu-chelation features have been investigated to evaluate extract antioxidant activity, as depicted in Table 2.

Table 2. Antioxidant activity expressed as DPPH· radical inhibition and Cu-chelation, with relative EC50, Trolox eq. and EDTA eq. values. Samples produced at 100 °C, 10 min.

	DPPH· Inhibition		**Cu-Chelating Activity**	
Matrix	**EC50** *(µg/mL)*	Trolox eq. *(µmolTE/gext)*	EC50 *(µg/mL)*	EDTA eq. *(µmolEDTA/gext)*
F-PP	2.30	3.30	88.60	513.66
D-PP	5.30	1.43	85.90	529.81

No pronounced differences in the antioxidant and Cu-chelating powers of the two pilot scale PP extracts were detected (Table 2), although F-PP displayed slightly better antioxidant power than D-PP (3.30 *vs.* 1.43 Trolox eq.), while the latter exhibited slightly higher Cu-chelating power (529.81 *vs.* 513.66 EDTA eq.). However, considering the negligible gap between the observed values, both of the recovered products can be considered promising for further valorization studies. Thus, a deeper investigation, using a biological assay, was conducted, details can be found in Section 3.3.

3.2.2. Fresh and Dried Pomegranate Peels: Energy Consumption

So far, all of the collected data confirm that the MAE of PP (100 °C, 10 min) provides a valuable extract in terms of the recovered metabolites and their antioxidant activity. The previous comparisons have aimed to define which matrix can be suitably exploited in a valorization protocol. For this

 doi:10.20944/preprints202308.0064.v1

purpose, an additional evaluation, this time of energy consumption, that evaluated the impact of the extraction and the dehydration steps on TPC yields was performed. Polyphenols were adopted as the reference compounds as they are the most comprehensively available parameter. The elements used to calculate *Polyphenol Energy Efficiency*, expressed as W/mgGAE, are reported in Table 3.

Table 3. Scaled-up MAE (100 °C, 10 min) of F-PP and D-PP: Polyphenol Energy Efficiency calculation as a function of yields and energy consumption.

	D-PP	**F-PP**
Dry yield *(%)*	67.20	20.18
TPC Yield *(mgGAE/gMatrix)*	175.64	48.99
Energy Consumption *(kW)**	1.94**	0.97
Polyphenols Energy Efficiency *(W/mgGAE)*	**11.07**	**19.72**

*Energy required by the scaled-up protocol to heat and cool the MW reactor;. **Energy required for drying procedure is added for D-PP.

It is worth noting that, although the dehydration step in producing D-PP requires extra energy intake (approx. 0.98 kW), the enhanced TPC yield (175.64 *vs.* 48.99 mgGAE/gMatrix) means that the resulting *Polyphenol Energy Efficiency* for D-PP is 11.07 W/mgGAE, which is a Watts saving of more than 55% per mgGAE compared to F-PP.

Thus, considering the characterization profile and the energy required to recover the extract, D-PP is more suitable as a feedstock for valorization by means of MAE.

3.2.3. Nanofiltration: Water Recovery and Metabolite Concentration

The extract produced via the optimized MAE of D-PP (100 °C, 10 min) was considered for membrane filtration using a nanofiltration protocol (NF, 150–300 Da). This downstream strategy leads to the recovery of large quantities of water, while also concentrating the final active product at the same time, dramatically reducing the need for time- and energy-consuming work-up procedures.

NF was performed on 2 L of D-PP extract mixture using a pilot membrane filtration system (PB100, Hydro Air Research Srl) equipped with a DKU 1812 NF membrane (filtering area: 0.38 m^2, cut-off range: 150–300 Da), that collected the concentrated metabolites in the retentate and the removed water in the permeate stream. The extract was concentrated 3.3 times (from 18.99 mg/mL to 62.86 mg/mL) after 25 min of processing, with an overall water recovery of approx. 75%. In addition, NF treatment partially enhanced product selectivity towards active metabolites, due to the partial removal of salts and monosaccharides into the permeate fraction (See Figure 7).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

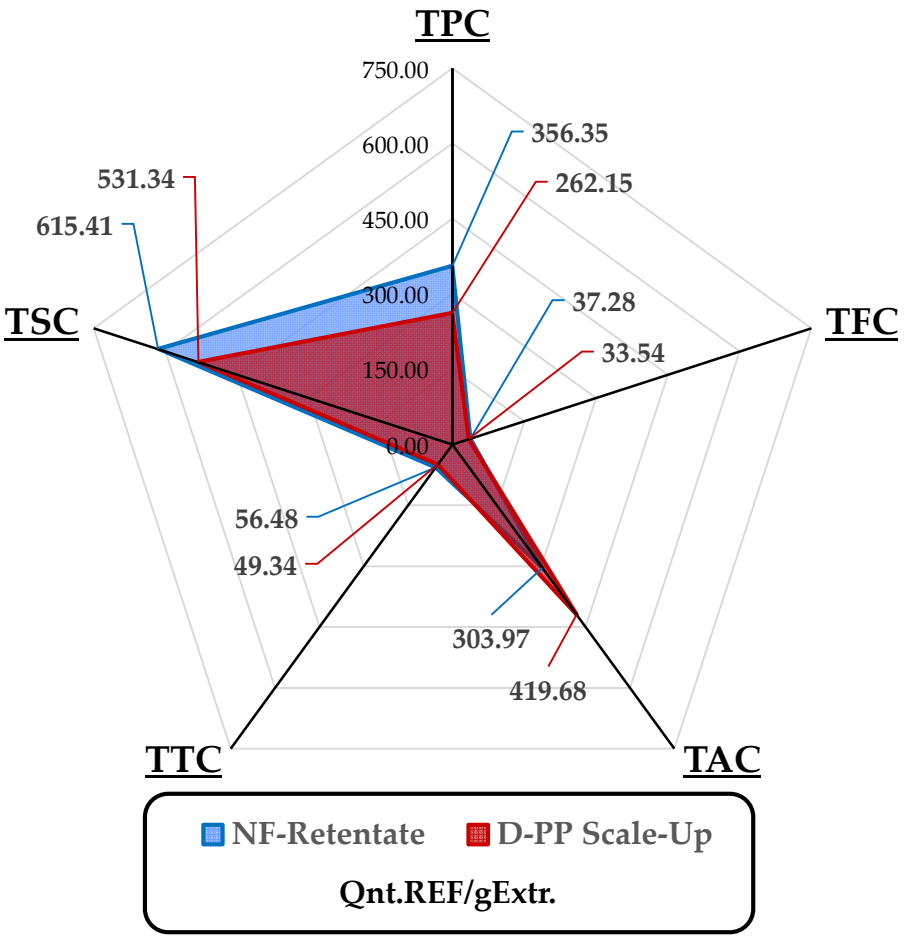


Figure 7. Main components of quali-quantitative analysis for MAE scale-up of D-PP and its NF-retentate. Selectivity is reported per gram of extract, reporting total weight of polyphenols, flavonoids, anthocyanins, tannins and sugars according to the adopted reference. In detail, TPC: mgGAE; TFC: mgQE; TAC: μgCy3G; TTC: mgGAE; TSC mgGLU.

Although no significant difference in the contents of total tannins and flavonoids was observed, as can be appreciated in Figure 7, NF increased the retentate's TPC concentration by more than 35%, compared to the D-PP extract, although there was also a slight contraction in TAC. Furthermore, the membrane concentration protocol also increased the antioxidant activity of the NF-retentate, which reached 554.33 μmolEDTA/gExtr, compared to the initial 529.81 μmolEDTA/gExtr of the D-PP scaled-up extract, and 3.04 *vs.* 1.43 μmolTE/gExtr. Moreover, the effective recyclability of the aqueous solution recovered in the permeate stream was tested, and it was found that the process negligibly affected final product quality.

For the sake of comparison, the efficiency of the MW-assisted pilot-scale protocol for D-PP extraction was evaluated against an hydroalcoholic process that was adopted as a benchmark (section 2.3). The percentage variations in MAE compared to the conventional protocol are depicted in **Figure 8**, where negative percentages represent values superior to those of the benchmark. The simple MAE of D-PP does not outperform the reference, except for TSC and Cu-chelating activity values (1.2- and 7-fold, respectively). Nevertheless, the additional, downstream NF step dramatically enhances the value of the final product, overtaking the hydroalcoholic extract's TPC and DPPH· inhibition values, in the last case by more than 28%. The remaining parameters, as seen in Figure 8, approach those of the reference, after a concentration increase of 9.7 and 4.5 % according to TFC and TTC, respectively.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

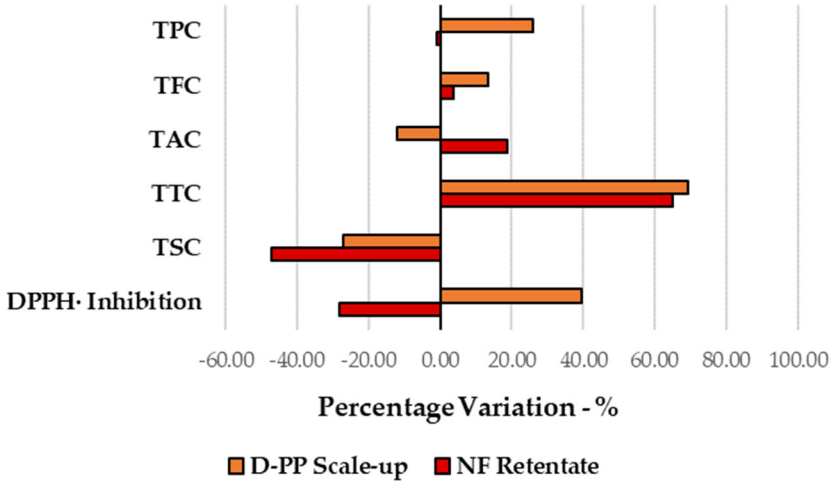


Figure 8. Comparison with hydroalcoholic benchmark extraction for D-PP scaled-up extraction and the related NF retentate. Percentage variations are calculated using conventional benchmark as the reference (*y*-axis).

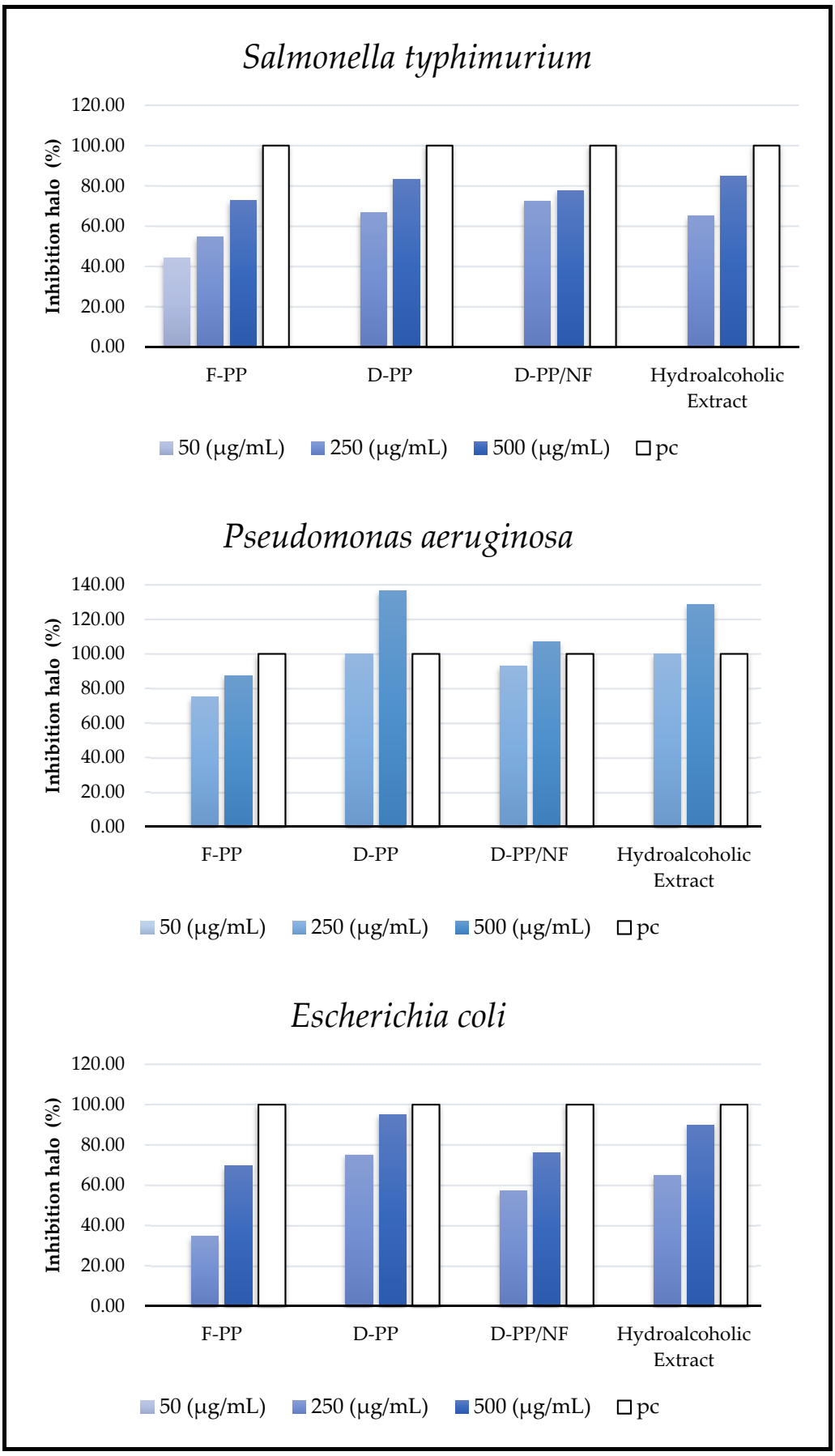
3.3. Biological Activity of PP Extracts

PP extracts have been used in traditional medicine for centuries because of their various health benefits. A wide range of bioactive compounds, including polyphenols, flavonoids, tannins, anthocyanins, and ellagitannins, contribute to their biological activity which includes antioxidant, anti-inflammatory, antimicrobial and anti-cancer properties. [45] In this work, we have assessed the main PP extract's antimicrobial properties against various bacteria and its anti-cancer activity, in terms of its potential to inhibit the growth of cancer cells. In the above-mentioned tests, we explored the products that were recovered from the optimized protocol and then characterized; the scaled-up extracts F-PP, D-PP, D-PP/NF together with the hydroalcoholic benchmark.

3.3.1. Antibacterial Activity of PP Extracts

Antibacterial activity against common Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis* and *Listeria monocytogenes*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) pathogenic bacterial strains was evaluated. Figure 9 shows the activity determined using a disc diffusion assay (example in Figure A1), normalized on the positive control (as 100%). The size of the inhibition halo is proportional to the susceptibility of the organism to the tested compound, meaning that the larger the inhibition zone is, the more the bacteria is susceptible to a particular extract. Complete data are reported in Appendix A (Table A1).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

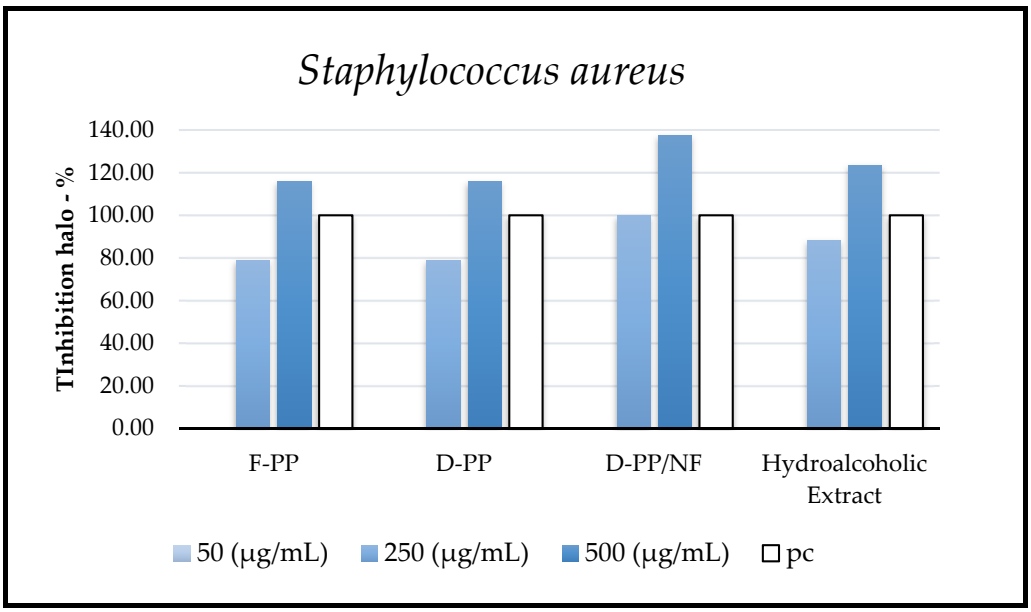


Figure 9. Antimicrobial activity of PP extracts against pathogens (mm). pc = positive control. Reported data are normalized on pc.

According to the results presented in Figure 9, the majority of pathogenic strains appeared to be sensitive to the PP extracts to some extent. The exceptions are *Listeria monocytogenes* and *Bacillus subtilis*, since no inhibition halos were detected with any of the four samples tested at any applied concentration (thus they are not reported in Figure 9). Of the tested pathogenic bacteria, only *S. typhimurium* was affected by F-PP at the lowest tested concentration (50 µg/mL) with an inhibition halo of 9.7 ± 0.6 mm. As can be seen in Figure 9, *S. aureus* was the most sensitive to all of the tested samples, as the inhibition halos of the PP extracts, at a concentration of 500 µg/mL, were 21 ± 0.6 mm and 22 ± 1.2 mm in size. Moreover, it worth mentioning that the PP extracts showed greater inhibition activity than the positive control. The same effect can be observed at the lower sample concentration (250 µg/mL), where *S. aureus* was the most inhibited bacteria (15 ± 0.6 mm to 16 ± 0.7 mm), although there is no significant difference in inhibition compared to the other three Gram-negative bacteria.

The obtained results can be compared with the work of Emam-Djomeh *et al.*, [46] who investigated the antimicrobial activity of PP extracts towards *E. coli* O157:H7 and *S. aureus*. According to their results, *S. aureus* was more sensitive than *E. coli* as the MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) of the samples for *S. aureus* were 125 ppm and 250 ppm, respectively, while for *E. coli*, the MIC and MBC were 250 ppm and 500 ppm, respectively. Differences in cell-surface hydrophobicity, [47] the higher complexity of the double membrane enveloping Gram-negative bacteria and the solubility of the extracts in the lipid phase of the membrane are some of the factors that have been suggested as being responsible for the differences in the antibacterial activity of the PP extracts. In comparison, Gram-positive cells have single layer of glycoprotein/teichoic acid, and even though it is thicker than in Gram-negative cells, it clearly does not provide enough protection to cells.

Research by Fabrizio Ferrazzano *et al.*, [48] has demonstrated that 10 and 15 µg/mL hydroalcoholic PP extracts effectively inhibited the growth and survival of the *S. mutans* ATCC 25,175 strain and the *R. dentocariosa* clinical isolate. In comparison, Alexandre *et al*., [49] have investigated the effect of the high pressure, enzymatic extraction of pomegranate by-products on antimicrobial activity against five Gram-positive (*B. cereus, S. aureus, Methicillin-resistant S.aureus, L. innocua and L. monocytogenes*), and three Gram-negative (*S. enteritidis, P. aeruginosa and E. coli*) food pathogenic bacteria, and five bacteria used as markers of beneficial gut microbiota, *i.e*. Lactic Acid Bacteria (LAB) (*L. plantarum, L. rhamnosus, L. acidophilus* and two strains of *B. animalis*). All of the tested PP extracts demonstrated selective antimicrobial activity against pathogenic bacteria without affecting the beneficial ones. Unlike our results, in Figure 9, Alexandre *et al.*, [49] showed that *P. aeruginosa* was more sensitive to the peel extract than all of the other tested microorganisms. In the case of LAB, only

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

small inhibition halos were observed for *L. rhamnosus* and *L. acidophilus*, which is related with the fact that LAB can metabolize phenolic compounds into volatile phenols, whereas the sugar moieties of anthocyanins may be used as an energy source. [50,51] Furthermore, the research of Alexandre *et al.*, [49] and Dahham *et al.*, [52] showed that *B. cereus, E. coli, S. aureus* and *P. aeruginosa* are affected by the PP extract. Ismail *et al.*, also reported that this product displayed inhibitory effects against *B. subtilis, S. aureus, P. aeruginosa, E. coli and S. typhimurium*. [53] However, our results are not in complete agreement with those of Ismail *et al.*, (2016) and Alexandre *et al.*, (2018), because B. *subtilis and L. monocytogenes* were not affected by the tested extract in our work. The observed differences in activity in studies may be partially explained by variations in the phenolic contents of the extracts, the antimicrobial procedures adopted in the tests and strain sensitivity as well as by differences in the strains used.

3.3.2. Antiproliferative Activity of PP Extracts

The antiproliferative activity of four PP extracts (scaled-up F-PP, D-PP, D-PP/NF together with the hydroalcoholic benchmark) has been evaluated, in experiments that investigated their ability to inhibit the growth of HeLa carcinoma cells. The cytotoxicity of the prepared samples was measured using the CellTiter AQueous One Solution Cell Proliferation Assay. Results are expressed as the cell viability (%) of treated cells against controls (non-treated cells), and are shown in Figure 10**.**

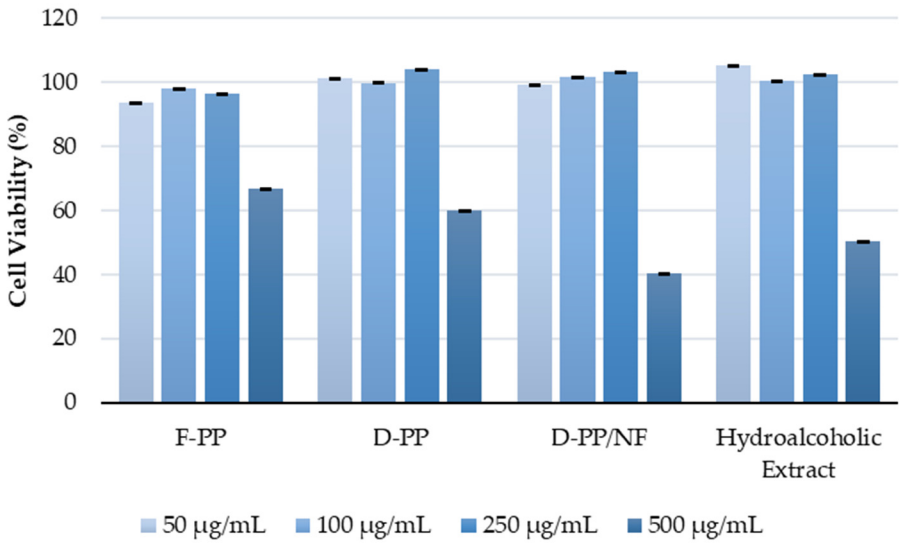


Figure 10. Viability of HeLa cells treated with four PP extracts for 72 h in the range of concentration from 50 µg/mL to 500 µg/mL assessed using the CellTiter AQueous One Solution Cell Proliferation Assay. Cell viability (%) is expressed as percentage of treated cells versus control cells.

All of the tested extracts display antiproliferative activity when cells are treated with the highest concentration of extracts (500 µg/mL), being the most pronounced in D-PP/NF with cell viability of 40.21%.. On the other hand, the lowest impact on cell growth was observed with F-PP; 66.84% cell viability after 72 hours of treatment at the highest tested concentration. Similar activity has already been reported in the literature, mostly for ethanol PP extracts, and in some cases, it is related to cell death as well as with cell-cycle arrest. For example, Keta *et al.*, have investigated the effects of an aqueous-ethanol PP extract on various human cancer cell lines (HTB140, HTB177, MCF7, HCT116) at a broad range of concentrations (15.63, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL). [54] They observed that the samples obtained using 50% ethanol in a US bath expressed selective cytotoxicity to cancer cells. Three cancer cell lines (HTB177 < HCT116 < MCF7) were considered to be sensitive to the obtained product, with MCF7 cells being the most sensitive. Work by Keta *et al.*, [54] has reported that the cytotoxic effect was observed at a lower concentration than that used in our work, with this

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

difference possibly being related to different types of extraction procedure used and therefore the different profiles of biologically active substances in the products. Similar results were observed with a methanolic extract of PP by Modaeinama *et al.*, [55]. Cytotoxicity (0, 5, 20, 100, 250, 500, 1000 µg/ml) was evaluated in MTT assays on lung non-small cell cancer (A549), breast adenocarcinoma (MCF-7), ovarian cancer (SKOV3) and prostate adenocarcinoma cells (PC-3). The samples reduced cell viability to values below 40%, even at the lowest doses, with EC_{50} values of below 5µg/ml, which is significantly lower than those observed here in HeLa cells. MCF-7 breast adenocarcinoma cells were the most responsive to the antiproliferative effects of the PP extracts, as was reported in Keta *et al.*. [54] The potential anti-cancer activity of PP extracts on the growth and cell-death mechanisms of chronic myeloid leukemia (CML) cells (K562) has also been investigated by Asmaa *et al.*, who observed that the ethanolic samples inhibited the growth of K562 cells, mainly via cell-cycle arrest and apoptosis induction, but at a lower rate. [56] One recent *in-vitro* study by Nasr *et al.*, performed against the liver cancer cell line (HepG2), confirmed the dose-dependent cytotoxicity of the tested extracts, which led to cell-cycle arrest and cell death. [57] The same group also compared the extracts of pomegranate seed and peels, and demonstrated that cell inhibition was much more effective in the first case.

In order to deepen our knowledge of the potential anti-cancer activity of the explored samples, the "colony formation test" or clonogenic assay was exploited, in this work, as another *in-vitro* method. It is based on the ability of a single cell to grow into a colony. Clone formation is, in some way, a property of unlimited growth, which is a special feature of tumor cells, meaning that the clonogenic assay may serve as a good indicator of the antitumor potential of the tested compounds. PP extracts were analyzed on HeLa cells treated with 500 µg/mL of the samples indicated in Figure 10. After 19 days of *in-vitro* cultivation, colonies became visible and were then colored with 0.5% crystal-violet, counted and photographed, as can be seen in Figure 11.

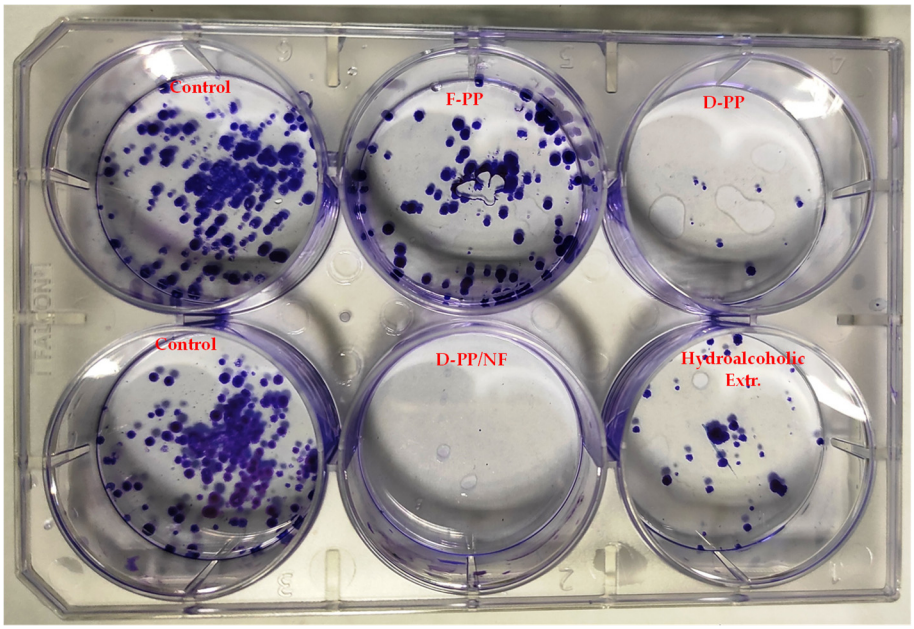


Figure 11. Result of clonogenic analysis after treatment with PP extracts at a concentration of 500 µg/mL.

Based on the number of colonies counted, the plating efficiency (PE) and surviving fraction (SF) were calculated for the tested compounds (Table 4), according to the equations in the protocol reported by Franken *et al.* [37] A higher SF value means that a higher colony-forming ability is maintained after treatment with the extract, with this ability potentially being related to that compound having less pronounced cytotoxic efficacy.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

Table 4. Surviving fraction (SF) for HeLa cells treated with PP extracts (500 μg/mL).

	F-PP	D-PP	D-PP/NF	Hydroalcoholic Extr.
SF	0.646	0.074	0	0.274

Table 4 shows the SF values of the treated HeLa cells. A higher value here (~1) indicates higher colony-formation ability after treatment. It is evident that there are differences in the SF values of the four tested extracts. The colony-formation ability depends on the type of sample extract used, with D-PP/NF seeming to be the most potent in terms of anti-cancer activity, as evidenced by its SF value of zero. The SF values for F-PP and the hydroalcoholic extract are higher than those of the other two evaluated compounds, which is consistent with their weaker inhibitory effect in the antiproliferative assay. Clonogenic analyses have also been performed by Keta *et al.*, but with significantly lower PP-extract concentrations (16 μg/mL), resulting in an SF of 0.74 in HTB177 cells, which were found to be more resistant than HTB140 cells, who displayed an SF of 0.47. [54] In their work, no colony formation was observed after treatment with 32 μg/mL of extract; clonogenic growth was completely inhibited.

It can be concluded that the results of the antiproliferative activity and clonogenic analyses of the tested PP extracts on the HeLa cell line indicates their potential to act as a natural anti-cancer product with biological activity. Other studies also have demonstrated that similar samples can inhibit the proliferation of cells in various types of cancer, including breast, prostate, colon, lung and skin cancers. In particular, the extract's bioactive compounds have been found to induce cell-cycle arrest, to decrease cell viability and promote apoptosis (programmed cell death), [45,54,56,57] through several mechanisms of action, such as antioxidant effects, anti-angiogenic effects, the modulation of signaling pathways, as well as showing chemopreventive potential.

3.4. Green Metrics & Energy Consumption of Extraction: A Preliminary Evaluation

Green metrics play a crucial role in evaluating environmental impact, serving as valuable tools to assess the efficiency and sustainability of chemical processes. Three of the main green metrics, RME, E-Factor and PMI (PME) have been exploited to evaluate the proposed MAE approach for D-PP valorization followed by the NF step, adopting the hydroalcoholic benchmark protocol as a reference. The values obtained using Equations (1)–(4) (section 1) are reported in Table 5.

Table 5. Green metrics for MAE of D-PP; comparison of hydroalcoholic benchmark, D-PP scale-up and its relative NF. Green bars: metrics considered directly proportional to sustainability; red bars: metrics considered inversely proportional to sustainability.

	RME (%)	E-Factor	PMI	PME (%)
Hydroalcoholic Benchmark	24.72	30.38	44.22	2.26
D-PP Scale-up	17.56	0.75	54.21	1.84
D-PP Scale-up NF	16.80	0.91	18.03	5.55

Firstly, RME can be used to describe how the matrix is exploited by the process, thus generating lower amounts of waste (see Equation (1)). In this case, the conventional protocol shows the best result, with an approx. 7%-point gap to the enabling technologies. However, RME does not consider solvents and their involvement in waste generation. For this reason, the E-Factor was considered since it includes solvents in the calculation (see Equation (2)). It worth remembering that lower values indicate a more sustainable process with minimal waste generation. According to the E-Factor values, the process intensification of MAE and MAE/NF is dramatically more environmentally friendly than the conventional protocol, although the E-Factor can provide bias to the evaluation of aqueous extractions as it excludes water. Hence, PMI and, the more intuitive, PME were considered to be the most objective green metrics (see Equations (3) and (4)). [21] In this evaluation, the conventional and MW-assisted processes are too close to call (only 0.42% points of difference), whilst the MAE-NF

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 1 August 2023 doi:10.20944/preprints202308.0064.v1

strategy was found to be the most sustainable protocol, as it more than doubled the PME value of the hydroalcoholic process. This result can be easily explained by the capacity to recycle approx. 75% of the overall water amount exploited.

The utilization of green metrics assumes a pivotal role in providing a guide for the design and optimization of sustainable processes. More environmentally viable practices can be adopted, achieving the objectives of waste minimization, resource conservation, and the reduction of the overall environmental impact associated with chemical processes.

Energy consumption, already assessed in Section 3.2.2. (D-PP/F-PP comparison), is a rational addition to the green metrics evaluated so far, and so was tentatively extended to MAE scaled-up/NF. The whole process was considered, including downstream elements, when evaluating the overall consumption and the relative energy distribution of each step (see Figure 9).

As expected, the lyophilization procedure has the largest contribution to the global energy intake, for both dry and fresh peels. On the other hand, this factor can be dramatically reduced thanks to the concentration of the extract, performed using NF, in the case of D-PP. Furthermore, this strategy reduces the energy consumption by more than 4-fold (3.51 *vs*, 14.57 kW). As depicted in Figure 12 (donut chart detail), membrane filtration is an extremely energy-efficient solution, accounting for a minimal part of the requirements of the whole process.

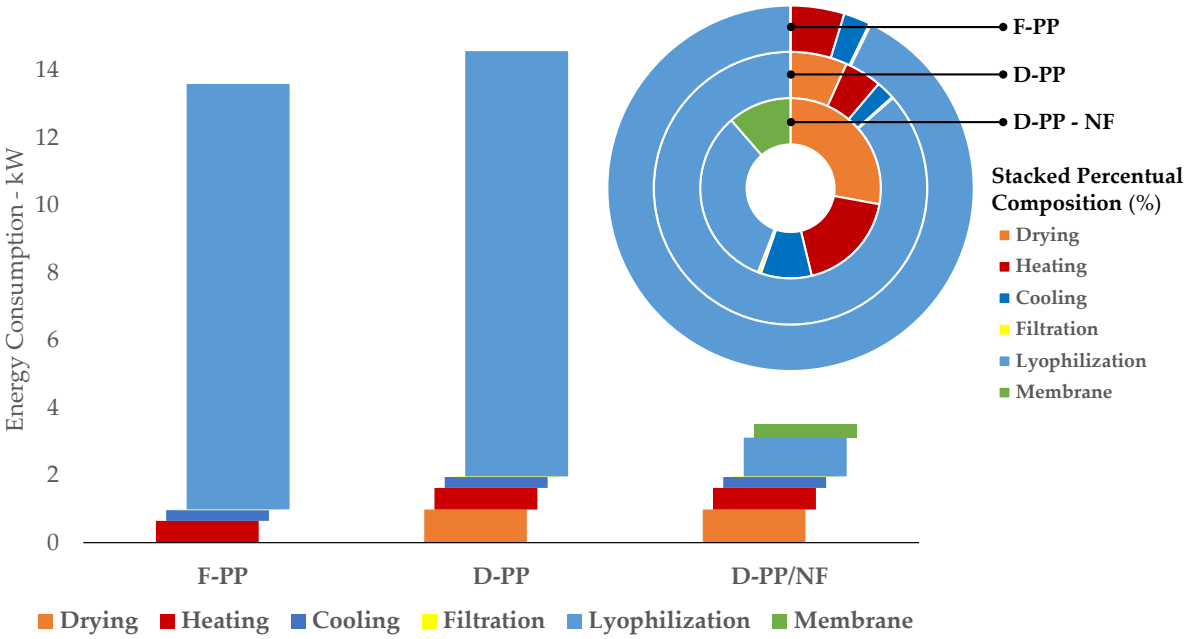


Figure 12. Energy Consumption and relative distribution. All data refer to scaled-up extractions.

Obviously, the removal of intake water can also be considerably decreased using other approaches, such as a spray-drying system. However, despite possessing a lower energy/evaporation ratio, the technology usually requires excipients to maintain good product recovery, which reduces metabolite selectivity and thus extract activity. Lastly, it is possible to state that, in general, the concentration of an extract by NF has beneficial effects on every type of de-watering technique. In fact, it is possible to refine the *Polyphenol Energy Efficiency* calculation, obtaining 277.32, 82.92 and 20.92 W/mgGAE, respectively for F-PP, D-PP and D-PP/NF. Thus, it is possible to state that, with the same amount of energy, polyphenol productivity can be approximately quadrupled in the case of the MW assisted PP-valorization process followed by an NF step.

5. Conclusions

The "zero-waste economy" concept, which aims to reduce waste and promote the efficient use of resources, perfectly meets the urgency of (food)waste-valorization strategies via the design of protocols that can exploit those residues as a resource for new products, rather than simply

discarding it. Furthermore, it is necessary to emphasize that food waste can be an outstanding source of bioactive compounds, including antioxidants and phenols. Pomegranate (*Punica granatum* L.) production is estimated to be about 8.1 million t/y, with related peel generation being of approx. 3.6 million tons, making it a huge environmental problem and economic loss. In this work, an MAE protocol has been optimized by evaluating the recovery of several components, such as polyphenols, anthocyanins, flavonoids, tannins and sugars, and their antioxidant and copper-chelating activity.

During the screening, matrix nature was explored, in addition to time and temperature variations, as we investigated how product quality and yield are affected by fresh and dried peels, as the latter was found to be the most promising feedstock. The study also involved the evaluation of potential water recycling, performed by means of a nanofiltration approach, which led to overall water reuse of approx. 75%. An evaluation of energy consumption (20.92W/mgGAE) and common green metrics gave encouraging results in terms of the sustainability of the non-conventional valorization strategy *vs.* the conventional approach (PME: 5.55% and 2.26%, respectively).

The screening also involved a preliminary transposition from lab-scale to a scaled-up protocol, from 1 to 20 grams, as a feasibility test, paving the way for further piloting considerations in view of the enormous amount of waste produced yearly.

The product furnished by the tuned extraction protocol can boast of the following contents per gram of PP extract: TPC: 356.35 mgGAE; TAC: 303.97 μgCy3G; TFC: 37.28 mgQE; TTC: 56.48 mgGAE; and, TSC: 615.41 mgGLU. In addition, Cu-chelation was 529.81 μmolEDTA and antioxidant activity was 1.43 μmolTE (according to the adopted reference). The biological activity of the recovered PP extracts has also been studied. The samples possess antimicrobial activity in laboratory studies, but further research is needed to determine their effectiveness for potential applications in the food, cosmetics and even pharmaceutical industries. While findings on the extracts' antiproliferative activity, *i.e.* the anti-cancer activity of PP extracts, are also promising, it is important to note that research on the anti-cancer activity of pomegranate extracts is still evolving, and more studies, including clinical trials, are needed to determine its efficacy, optimal dosage and potential interactions with other cancer therapies. Nevertheless, pomegranate peel is a valuable by-product whose extracts, especially those achieved using green extraction, deserve further investigation due to numerous benefits of the approach.

Author Contributions: "Conceptualization, E.C.G., M.B., K.R. and G.Cr.; methodology, G.G., Z.J. and L.G.; validation, K.R., K.M. and G.G.; formal analysis, Z.J., K.M. and L.G.; investigation, G.G., G.Ca., Z.J. and E.C.G.; data curation, K.R., E.C.G. and M.B.; writing—original draft preparation, G.G., G.Ca. and K.R.; writing—review and editing, G.Cr. and G.G.; supervision, G.Cr. and K.R..

Data Availability Statement: Not applicable.

Acknowledgments: The University of Turin is acknowledged for the financial support (Ricerca Locale 2022)

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Figure A1. Representative result of a disc diffusion assay on S. aureus treated with PP extract (50 µg/mL, 250 and 500 µg/mL) compared to negative and positive controls.

Table A1. Antimicrobial activity of PP extracts against pathogens (mm). pc = positive control; nc = negative control.

	Conc. (µg/mL)	***S. typhimurium***	**S.D.**	***P. aeruginosa***	**S.D.**	***E. coli***	**S.D.**	***S. aureus***	**S.D.**	***L. monocytogenes***	**S.D.**	***B. subtilis***	**S.D.**
F-PP	50	9.7	0.6	0	0	0	0	0	0	0	0	0	0
	250	12	0.6	12	0.6	8	2.1	15	0.6	0	0	0	0
	500	16	1.5	14	1	16	1.5	22	1.2	0	0	0	0
	pc	22	1.2	16	1.2	23	0.6	19	1	17	0	16	1.2
	nc	0	0	0	0	0	0	0	0	0	0	0	0
D-PP	50	0	0	0	0	0	0	0	0	0	0	0	0
	250	12	0	11	1.2	15	3.1	15	0.6	0	0	0	0
	500	15	1.2	15	2.1	19	1.2	22	1.2	0	0	0	0
	pc	18	3.5	11	1.2	20	1	19	1	16	2.5	17	0
	nc	0	0	0	0	0	0	0	0	0	0	0	0
D-PP/NF	50	0	0	0	0	0	0	0	0	0	0	0	0
	250	13	0	13	1.2	12	1	16	0.7	0	0	0	0
	500	14	0.6	15	1.2	16	2.1	22	1.2	0	0	0	0
	pc	18	0.6	14	1	21	1	16	0.6	15	0.6	16	1

	nc	0	0	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0	0	0	0	0
	250	13	0.6	14	0	13	1.2	15	0.6	0	0	0	0
Hydroalcoholic Extract	500	17	1	18	0.6	18	1.5	21	0.6	0	0	0	0
	pc	20	1	14	2.1	20	0	17	1	14	1.5	12	0.6
	nc	0	0	0	0	0	0	0	0	0	0	0	0

References

1. FAO. Technical Platform on the Measurement and Reduction of Food Loss and Waste. **2022**. Available online: https://www.fao. org/platform-food-loss-waste/en/(accessed on 6 May 2023).
2. Girotto, F.; Alibardi, L.; Cossu, R.; Food waste generation and industrial uses: A review. *Waste Manag.* **2015**, *45*, 32-41. Doi: 10.1016/j.wasman.2015.06.008
3. Calcio Gaudino, E.; Colletti, A.; Grillo, G.; Tabasso, S.; Cravotto, G. Emerging Processing Technologies for the Recovery of Valuable Bioactive Compounds from Potato Peels. *Foods* **2020**, *9*, 1598. Doi: 10.3390/foods9111598
4. Cravotto, C.; Grillo, G.; Binello, A.; Gallina, L.; Olivares-Vicente, M.; Herranz-López, M.; Micol, V.; Barrajón-Catalán, E.; Cravotto, G. Bioactive Antioxidant Compounds from Chestnut Peels through Semi-Industrial Subcritical Water Extraction. *Antioxidants* **2022**, *11*, 988. Doi: 10.3390/antiox11050988
5. Grillo, G.; Boffa, L.; Talarico, S.; Solarino, R.; Binello, A.; Cavaglià, G.; Bensaid, S.; Telysheva, G.; Cravotto, G. Batch and Flow Ultrasound-Assisted Extraction of Grape Stalks: Process Intensification Design up to a Multi-Kilo Scale. *Antioxidants* **2020**, 9, 730. Doi: 10.3390/antiox9080730
6. Ravindran R.; Jaiswal AK. Exploitation of Food Industry Waste for High-Value Products. *Trends Biotechnol*. **2016**, *34*(1), 58-69. Doi: 10.1016/j.tibtech.2015.10.008.
7. Chemat, F.; Vian, M.A.; Fabiano-Tixier, A.-S.; Nutrizio, M.; Jambrak, A.R.; Munekata, P.E.S.; Lorenzo, J.M.; Barba, F.J.; Binello, A.; Cravotto, G. A review of sustainable and intensified techniques for extraction of food and natural products. *Green Chem.* **2020**, *22*, 2325–2353. Doi: 10.1039/C9GC03878G
8. Calcio Gaudino, E.; Grillo, G.; Tabasso, S.; Stevanato, L.; Cravotto, G.; Marjamaa, K.; Pihlajaniemi, V.; Koivula, A.; Aro, N.; Uusitalo, J.; et al. Optimization of ultrasound pretreatment and enzymatic hydrolysis of wheat straw: From lab to semi-industrial scale. *J. Clean. Prod*. **2022**, *380* Part 1, 134897. Doi: 10.1016/j.jclepro.2022.134897
9. An, Q.; Li, L.; Liu, Y.; Wang, S.; Yuan, Q. Bioplastic production from food waste: A review. *J. Clean. Prod* **2021**, *290*, 125586. Doi: 10.1016/j.jclepro.2020.125586
10. Tabasso, S.; Mariatti, F.; Grillo, G.; Boffa, L.; Tibaldi, P.S.; and Cravotto, G. *Ind. Eng. Chem. Res* **2019,** *58*(20), 8578-8584, Doi: 10.1021/acs.iecr.9b01330
11. Ferdeș, M.; Zăbavă, B.Ș.; Paraschiv, G.; Ionescu, M.; Dincă, M.N.; Moiceanu, G. Food Waste Management for Biogas Production in the Context of Sustainable Development. *Energies* **2022**, *15*, 6268. https://doi.org/10.3390/en15176268;
12. Johnravindar, D.; Wong, J.W.C.; Dharma Patria, R.; Uisan, K.; Kumar, R.; Kaur, G. Bioreactor-scale production of rhamnolipids from food waste digestate and its recirculation into anaerobic digestion for enhanced process performance: Creating closed-loop integrated biorefinery framework. *Bioresour Technol.* **2022,** *360*, 127578. Doi: 10.1016/j.biortech.2022.127578.
13. Roger, A. S.; Green Chemistry: Principles and Case Studies. By Felicia A. Etzkorn. *Angew. Chem. Int. Ed*. **2021**, *60*, 513-1032. Doi: 10.1002/anie.202008458
14. Chemat, F.; Vian, M.A.; Cravotto, G. Green Extraction of Natural Products: Concept and Principles. *Int. J. Mol. Sci.* **2012**, *13*, 8615–8627. Doi: 10.3390/ijms13078615
15. Chemat, F.; Abert-Vian, M.; Fabiano-Tixier, A.S.; Strube, J.; Uhlenbrock, L.; Gunjevic, V.; Cravotto, G. Green extraction of natural products. Origins, current status, and future challenges. *Trends Anal. Chem*. **2019**, *118*, 248–263. Doi: 10.1016/j.trac.2019.05.037
16. Basak, S.; Annapure, U.S. The potential of subcritical water as a "green" method for the extraction and modification of pectin: A critical review. *Food Res. Int*. **2022**, *161*, 111849. Doi: 10.1016/j.foodres.2022.111849

17. Shitu, A.; Izhar, S.; Tahir, T.M. Sub-critical water as a green solvent for production of valuable materials from agricultural waste biomass: A review of recent work. *Global J. Environ. Sci. Manage*. **2015**, *1*, 255–264. Doi: 10.7508/gjesm.2015.03.008
18. Roger, A. S.; Metrics of Green Chemistry and Sustainability: Past, Present, and Future. *ACS Sustain. Chem. Eng* **2018,** *6*(1), 32-48. Doi: 10.1021/acssuschemeng.7b03505.
19. Curzons, A. D.; Constable, D. J. C.; Mortimer, D. N.; Cunningham, V. L. So you think your process is green, how do you know?—Using principles of sustainability to determine what is green–a corporate perspective, *Green Chem*., **2001**,*3*, 1-6. Doi: 10.1039/B007871I
20. Roger A. S.; The E Factor: fifteen years on, *Green Chem*., **2007**,*9*, 1273-1283. Doi: 10.1039/B713736M
21. Jimenez-Gonzalez, C.; Ponder, C. S.; Broxterman Q. B.; Manley J. B.; Using the Right Green Yardstick: Why Process Mass Intensity Is Used in the Pharmaceutical Industry To Drive More Sustainable Processes. *Org. Process. Res. Dev*. **2011**, *15*(4), 912-917. Doi: 10.1021/op200097d
22. Singh B.; Singh J. P.; Kaur, A.; Singh, N. Phenolic compounds as beneficial phytochemicals in pomegranate (Punica granatum L.) peel: A review, *Food Chem* **2018**, *261*, 75-86. Doi: 10.1016/j.foodchem.2018.04.039
23. Report by Louw, P.; article by Vanessa Barends-Jones, V. The Economic Contribution of South Africa's Pomegranate Industry. *Elsenburg Journal* **2021**. https://journals.co.za/doi/pdf/10.10520/ejc-agriprob-v18-n4-a23
24. Fahmy, H. A.; Farag, M. A. Ongoing and potential novel trends of pomegranate fruit peel; a comprehensive review of its health benefits and future perspectives as nutraceutical. J. *Food Biochem* **2022**, *46*, e14024. Doi: 10.1111/jfbc.14024
25. Panth, N.; Manandhar, B.; Paudel, K. R. Anticancer Activity of Punica granatum (Pomegranate): A Review. *Phytother. Res.* **2017**, *31*, 568– 578. Doi: 10.1002/ptr.5784
26. Zhang, Z.; Han, X.; Shi, J.; Xu, X. Review: Extraction of natural antioxidants from the matrices of plant foods. *J. Food Sci. Technol* **2018**, *55*(2), 387-399 Doi: 10.1533/9780857090751.2.506
27. Cano-Lamadrid, M.; Martínez-Zamora, L.; Castillejo, N.; Artés-Hernández, F. From Pomegranate Byproducts Waste to Worth: A Review of Extraction Techniques and Potential Applications for Their Revalorization. *Foods* **2022**, *11*, 2596. Doi: 10.3390/foods11172596
28. Hillis W.E.; T. Swain, T. The Phenolic Constituents Of Prunus Domestica. II-The Analysis of Tissues of the Victoria Plum Tree. *J. Sci. Food Agric*., **1959**, *10*, 135 – 144 Doi: 10.1002/jsfa.2740100211
29. Giusti, M.; Wrolstad, R.E. Acylated anthocyanins from edible sources and their applications in food systems. *Biochem. Eng. J.* **2003**, *14*, 217–225.
30. Peri, C.; Pompei, C. Estimation of Different Phenolic Groups in Vegetable Extracts. *Phytochemistry* **1971**, *10*, 2187–2189. Doi: 10.1016/S0031-9422(00)97216-9.
31. Aimone, C.; Grillo, G.; Boffa, L.; Giovando, S.; Cravotto, G. Tannin Extraction from Chestnut Wood Waste: From Lab Scale to Semi-Industrial Plant. *Appl. Sci.* **2023**, *13*, 2494. https://doi.org/10.3390/app13042494
32. Sen, S.; De, B.; Devanna, N.; Chakraborty, R. Total phenolic, total flavonoid content, and antioxidant capacity of the leaves of *Meyna spinosa* Roxb., an Indian medicinal plant. *Chin J Nat Med*. **2013,** *11*(2), 149-57. Doi: 10.1016/S1875-5364(13)60042-4.
33. Yemm, E.W.; Willis, A.J. The estimation of carbohydrates in plant extracts by anthrone. *Biochem J.* **1954,** *57*(3), 508-14. Doi: 10.1042/bj0570508.
34. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Food Sci. Technol*. **1995**, *28*, 25–30. Doi: 10.1016/S0023-6438(95)80008-5.
35. Locatelli, M.; Gindro, R.; Travaglia, F.; Coïsson, J.D.; Rinaldi, M.; Arlorio, M. Study of the DPPH-scavenging activity: Development of a free software for the correct interpretation of data. *Food Chem.* **2009**, *114*, 889–897. Doi: 10.1016/j.foodchem.2008.10.035.
36. Santos, J.S.; Brizola, V.R.A.; Granato, D. High-throughput assay comparison and standardization for metal chelating capacity screening: A proposal and application. *Food Chem*. **2017**, *214*, 515–522. Doi: 10.1016/j.foodchem.2016.07.091.
37. Franken, N.; Rodermond, H.; Stap, J; Haveman, J.; van Bree, C. Clonogenic assay of cells *in vitro*. *Nat Protoc,* **2006**, *1*, 2315–2319. Doi: 10.1038/nprot.2006.339.
38. Zin, M.M.; Anucha, C.B.; Bánvölgyi, S. Recovery of Phytochemicals via Electromagnetic Irradiation (Microwave-Assisted-Extraction): Betalain and Phenolic Compounds in Perspective. *Foods 2020*, *9*, 918. Doi: 10.3390/foods9070918.
39. Hayat, K.; Abbas, S.; Hussain, S.; Shahzad, S.A.; Tahir, M.U. Effect of microwave and conventional oven heating on phenolic constituents, fatty acids, minerals and antioxidant potential of fennel seed, *Industrial Crops and Products*, *140*, **2019**, 111610. Doi: 10.1016/j.indcrop.2019.111610.
40. Basak, S.; Annapure, U.S. The potential of subcritical water as a "green" method for the extraction and modification of pectin: A critical review, *Food Res. Int.*, **2022**, *161*, 111849, Doi: 10.1016/j.foodres.2022.111849;

41. Thani, N.M.; Kamal, S.M.M; Sulaiman, A.; Taip, F.S.; Omar, R.; Izhar, S. Sugar Recovery from Food Waste via Sub-critical Water Treatment, *Food Reviews International*, **2020**, *36*(3), 241-257. Doi: 10.1080/87559129.2019.1636815.
42. Makhafola, T.J.; Elgorashi E.E.; McGaw L.J.; Verschaeve L.; Eloff, J.N. The correlation between antimutagenic activity and total phenolic content of extracts of 31 plant species with high antioxidant activity. *BMC Complement Altern Med*. **2016,** *16*(1), 490. doi: 10.1186/s12906-016-1437-x.
43. Hider, R.C.; Liu, Z.D.; Khodr, H.H. Metal chelation of polyphenols. *Methods in Enzymology*. **2001**, *335*, 190-203. Doi: 10.1016/S0076-6879(01)35243-6
44. Vieira do Carmo, M.A.; Girotto Pressete, C.; Marques, M.J.; Granato, D.; Azevedo, L. Polyphenols as potential antiproliferative agents: scientific trends, *Current Opinion in Food Science*, **2018**, *24*, 26-35, Doi: 10.1016/j.cofs.2018.10.013.
45. Mo, Y., Ma, J., Gao, W., Zhang, L., Li, J.,Li, J. Zang, J. Pomegranate Peel as a Source of Bioactive Compounds: A Mini Review on Their Physiological Functions. *Front. Nutr*., **2022**, *9*, 887113. Doi: 10.3389/fnut.2022.887113.
46. Emam-Djomeh, Z.; Moghaddam, A.; Ali Yasini Ardakani, S. Antimicrobial Activity of Pomegranate (*Punica granatum* L.) Peel Extract, Physical, Mechanical, Barrier and Antimicrobial Properties of Pomegranate Peel Extract-incorporated Sodium Caseinate Film and Application in Packaging for Ground Beef, *Packaging Technology and Science,* **2015,** ***28***, 869-881. Doi: 10.1002/pts.2145.
47. Holley, R.A.; Patel, D. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicro-bials. *Journal of Food Microbiology,* **2005**, *22*(4), 273–292. Doi: 10.1016/j.fm.2004.08.006.
48. Ferrazzano, G.F.; Scioscia, E.; Sateriale, D.; Pastore, G.; Colicchio, R.; Pagliuca, C.; Cantile, T.; Alcidi, B.; Coda, M.; Ingenito, A.; Scaglione, E.; Cicatiello, A.G.; Volpe, M.G.; Di Stasio, M.; Salvatore. P.; Pagliarulo, C. *In Vitro* Antibacterial Activity of Pomegranate Juice and Peel Extracts on Cariogenic Bacteria. *Biomed Res Int.* **2017**, *2017*, 2152749. doi: 10.1155/2017/2152749.
49. Alexandre, E.M.C.; Silva, S.; Santos, S.A.O.; Silvestre, A.J.D.; Duarte, M.F.; Saraiva, J.A.; Pintado, M. Antimicrobial activity of pomegranate peel extracts performed by high pressure and enzymatic assisted extraction. *Food Research International.* **2019**, *115*, 167-176. doi:10.1016/j.foodres.2018.08.044.
50. García-Ruiz, A.; Bartolomé, B.; Martínez-Rodríguez, A.J.; Pueyo, E.; Martín-Álvarez, P.J.; Moreno-Arribas, M.V. Potential of phenolic compounds for controlling lactic acid bacteria growth in wine. *Food Control*, **2008**, *19*(9), 835-841. Doi: 10.1016/j.foodcont.2007.08.018.
51. Rodríguez, H.; Curiel, J.A.; Landete, J.M.; de las Rivas, B.; de Felipe, F.L.; GómezCordovés, C.; Mancheño, J.M.; Muñoz, R. Food phenolics and lactic acid bacteria. *International Journal of Food Microbiology*, **2009**, *132*(2), 79-90. Doi: 10.1016/j.ijfoodmicro.2009.03.025.
52. Dahham, S.; Ali, M.N.; Tabassum, H.; Khan, M. Studies on antibacterial and antifungal activity of pomegranate (*Punica granatum* L.). *Am-Euras. J. Agric. & Environ. Sci.,* **2010**, *9*, 273-281.
53. Ismail, T.; Akhtar, S.; Sestili, P.; Riaz, M.; Ismail, A.; Labbe, R.G.; Antioxidant, Antimicrobial and Urease Inhibitory Activities of Phenolics-Rich Pomegranate Peel Hydro-Alcoholic Extracts. *Journal of Food Biochemistry*, **2016**, *40*(4), 550-558. Doi: 10.1111/jfbc.12250.
54. Keta, O.; Deljanin, M.; Petković, V.; Zdunić, G.; Janković, T.; Živković, J.; Ristić-Fira, A.; Petrović, I.; Šavikin, K. Pomegranate (Punica granatum L.) Peel Extract: Potential CytotoxicAgent against Different Cancer Cell Lines. *Rec. Nat. Prod.* **2020,** *14*, 5326-339. Doi: http://doi.org/10.25135/rnp.170.19.11.1477.
55. Modaeinama, S.; Abasi, M.; Abbasi, M.M.; Jahanban-Esfahlan, R. Anti Tumoral Properties of *Punica Granatum* (Pomegranate) Peel Extract on Different Human Cancer Cells. *Asian Pac J Cancer Prev*, **2015**, *16*(14), 5697-5701. DOI:http://dx.doi.org/10.7314/APJCP.2015.16.14.5697.
56. Asmaa, M.J.; Ali, A.J.H.; Farid, J.M.; Azman, S. Growth inhibitory effects of crude pomegranate peel extract on chronic myeloid leukemia, K562 cells. *Int J App Basic Med Res* **2015**, *5*, 100-5. Doi:10.4103/2229-516X.157154
57. Nasr, M.; Naeem, S.A.; El-Shenbaby, I.; Mohamed, F.M.A.; Mahmoud, S.M.; Abuamara, T.M.M.; Abd-Elhay, W.M.; Elbayoumy, F.M.A.E.; Elkot, A.; Shikhon, T.; Abo-Akrab, M.; Doma, M.A.; Hasan, A. Pomegranate Seeds and Peel Ethanolic Extracts Anticancer Potentials and Related Genetic, Histological, Immunohistochemical, Apoptotic and Oxidative Stress Profiles: *In vitro* Study. *J Exp Pharmacol*. **2023**, *15*, 191-205. Doi: 10.2147/JEP.S404321.