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Antimicrobial and Antibiofilm Activities of Pomegranate Peel Phenolic Compounds: Varietal Screening Through a Multivariate Approach

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Abstract: Pomegranates are rich in phenolic compounds and known for their antioxidant, anti-inflammatory, and anticancer properties. The highest concentration of these compounds is found in the peel (exocarp and mesocarp), which constitutes about 50% of the whole fresh fruit. These bioactive phytochemicals exhibit a broad spectrum of antimicrobial effects against both Gram-negative and Gram-positive bacteria, as well as fungi. In the present paper, the chemical composition and antimicrobial activity of the peel (exocarp and mesocarp) from seven *Punica granatum* varieties (Wonderful, Mollar de Elche, Primosole, Sassari 1, Sassari 2, Sassari 3, and Arbara Druci) grown in Sardinia (Italy) were evaluated. Polar phenols, flavonoids, condensed tannins, and anthocyanin contents were evaluated by extraction with water at 20 and 40 °C. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) was used to characterize each variety according to the chemical composition of the pomegranate peel extracts (PPEs). The antimicrobial and antibiofilm activities of each PPE were further tested in vitro against *Staphyloccocus aureus*, *Listeria monocytogenes*, *Salmonella bongori*, *Escherichia coli*, *Lacticaseibacillus casei* Shirota, and *Limosilactobacillus reuteri*. Gram-positive species were more sensitive than Gram-negative to the extracts tested. Antimicrobial activity was shown against *S. aureus* and *L. monocytogenes* strains, whereas less, even no activity was found against *Sa. bongori* and *E. coli* strains. The PPEs from Mollar de Elche, Primosole, and Sassari 3 showed the highest antimicrobial activities at concentrations that varied from 0.19 to 1.50 mg/mL, with biofilm activity being reduced by more than 70%. These activities were positively related to the punicalagin, flavonoid, and chlorogenic acid content of the extracts. Finally, regarding the pro-technological bacterial strains, *La. casei* Shirota and *Li. reuteri* 17938 showed very low, even no sensitivity to the used of the specific PPEs with high concentrations. This study proposes a formulation of pomegranate peel extract that valorizes agro-industrial waste in the context of sustainability and circular economy. Pomegranate extracts

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should be considered potential sources of natural, plant-derived antimicrobials, providing an alternative to artificial antimicrobial products.

Keywords: *Punica granatum*; agrobiodiversity; OPLS-DA; punicalagin; *Staphyloccocus aureus*; *Listeria monocytogenes*

1. Introduction

The pomegranate (*Punica granatum* L.) is one of the oldest species of domesticated fruit. Originating in Iran and surrounding areas, this ancient fruit has spread across the world and been part of the human diet for around 5 000 years (Chandra et al., 2010). Its cultivation has been adapted to a wide range of environmental conditions, from the Mediterranean to desert climates, and it is presently produced at commercial levels in many areas of the world (Schwartz et al., 2009). The market demand for pomegranate fruit and its derived products, primarily in the form of juice, minimally processed fruits, jam, and dietary supplements, has increased greatly over the last decade. An important factor contributing to the expansion of its popularity, commercial production, and consumption habits has been the emergence of scientific evidence demonstrating the health-promoting benefits of this fruit (Kandylis and Kokkinomagoulos, 2020).

The pomegranate is rich in bioactive phytochemicals (present in different parts of the fruit) which are known for their antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. The pomegranate peel (exocarp and mesocarp), which constitutes about 50% of the whole fresh fruit, contains the highest concentration of phenolic compounds, mainly hydrolyzable ellagitannins, anthocyanins, and flavonoids (Akhtar et al., 2015). As a consequence, this part of the pomegranate, considered a by-product in the agri-food sector, should instead be designated a co-product from which a host of chemical compounds can be extracted for numerous applications as food additives, nutraceuticals, and supplements in the pharmaceutical, food, and cosmetics industries (Singh et al., 2018; Puneeth and Chandra, 2020; Kaderides et al., 2021, Gigliobianco et al., 2022).

Hydrolyzable ellagitannins (ellagic acid, punicalagin, punicalin, and gallic acid) are the predominant phenolic compounds in pomegranate peel, and they are also the constituent phytochemicals exhibiting the highest antioxidant capacities (Gigliobianco et al., 2022). For example, the chemical structure of punicalagin, which contains sixteen phenolic hydroxyl groups, underlies its ability to scavenge free radicals, as well as its anti-proliferative, anti-inflammatory, hepatoprotective, and antigenotoxic activities (Oudane et al., 2018). Ellagic acid is the main molecule present in pomegranate peel exhibiting anticancer activity (Puneeth and Sharath, 2020), whereas the anthocyanins are known for their antioxidant, anti-inflammatory, and chemopreventative properties (Li et al., 2017). Moreover, the magnitude of the antioxidant and antitumor activities of pomegranate peel extract is stronger than the sum of the individual activities of its constitutive bioactive molecules, indicating a possible synergistic effect resulting from the mixtures of phenolic compounds present in the pomegranate (Orgil et al., 2014; Kandylis and Kokkinomagoulos, 2020).

Infectious diseases and food decomposition caused by pathogenic microorganisms are two of the principal causes of morbidity and death worldwide (Endo et al., 2018; Celiksoy and Heard, 2021). Notably, food poisoning is predominantly linked to bacterial contamination by Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*. However, Gram-positive bacteria, including

Staphylococcus aureus and *Bacillus cereus*, have also been reported as the causal agents of food spoilage (Mostafa et al., 2018).

The widespread use of antibiotics to control life-threatening infectious diseases in humans and animals has resulted in the rise and spread of antibiotic-resistance mechanisms among bacterial pathogens. Moreover, the phenomenon of recalcitrant infections, involving the growth of bacteria or fungi in biofilms, has given rise to a second major challenge in dealing with microbial resistance (Slobodníková et al., 2016). Biofilms play a central role in the phenomenon of multi-drug resistance as they shield the microbes behind a protective coat made of extracellular polymeric substances (Bakkiyaraj et al., 2013). Furthermore, the antibiotics available at present are ineffective at treating biofilm-related infections due to their high minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), meaning that biofilm-related infections have the potential to result in in vivo toxicity (Roy et al., 2018).

The search for natural antimicrobials, especially plant-derived compounds, as an alternative to artificial antimicrobial products for the treatment of certain enteric infections is presently enjoying a surge in research attention (Pai et al., 2011; Xu et al., 2017). The peels of *P. granatum* present promising antimicrobial activity against antibiotic-resistant microbial strains such as methicillin-resistant *Staphylococcus aureus* (Pagliarulo et al., 2016; Xu et al., 2017; Singh et al., 2019). Several studies have reported pomegranate extracts to exhibit a broad spectrum of antimicrobial effects against both Gram-negative and Gram-positive bacteria, as well as fungi (Singh et al., 2019; Chen et al., 2020; El-Beltagi et al., 2022). Different parts of pomegranate fruit (peel, seeds, juice, and whole fruits) were tested for their antimicrobial activity against a range of bacterial species (*Staphylococcus aureus, Bacillus cereus, B. subtilis, B. coagulans, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa*), and the highest inhibitory values were consistently obtained using the peel extracts (Singh et al., 2019; Chen et al., 2020).

In order to recover as many antioxidants from a plant source as possible, the most appropriate extraction solvent must be used. Methanol or hydroalcoholic solutions are effective solvents for phenolic compounds, and they are generally the solvent of choice in the laboratory; however, for large scale industrial applications, the possibility of using deionized water as a more economically sustainable and eco-friendly alternative should be considered (Russo et al., 2018; Wu et al., 2021; El-Beltagi, 2022). Wang et al. (2011) reported that water extraction at 40 ℃ for 4 h was an efficient medium for extracting the antioxidants from pomegranate peel, due to the polar nature of these compounds, and produced comparable results to methanol. They concluded that water should be the solvent of choice, especially when considering the significant impact of methanol in terms of cost and toxicity. Another advantage of using water as extract solvent is that the antimicrobial properties of the resulting pomegranate peel extract can be tested directly without the need for further steps to remove alcoholic solvent (e.g., by vacuum distillation or freeze-drying).

Aqueous pomegranate peel extract was tested as an additive to extend the shelf-life of meat products. It was found to retard protein and lipid oxidation and induce antimicrobial activity against a range of pathogenic strains (Pirzadeh et al., 2021). Another study used extracts obtained using boiling water, and demonstrated them to exhibit antimicrobial activity in vitro against microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Salmonella typhimurium* at levels comparable to those of acetone and ethanol extracts (Nuamsetti et al., 2012). In a recent paper, Wu et al. (2022) demonstrated that aqueous extracts of pomegranate peels had a significant inhibitory effect on the growth of two *Salmonella enterica* strains. Moreover, El-Beltagi et al. (2022) confirmed that water extracted pomegranate peel extract (PPE) had a high antimicrobial activity compared with ethanol extracted PPE against *S. aureus* and *E. coli*. As reported by Melgarejo-Sánchez et al. (2021), few studies have compared the phytochemical composition of different

varieties of *P. granatum* or evaluated the nutraceutical effects as a function of genotype. Since the number of bioactive compounds is known to distinguish the different pomegranate varieties, the authors recommended carrying out specific studies to elucidate the biological activities of individual compounds and their synergistic actions related to genetic variability.

On these bases, the aim of the present work was to characterize the chemical composition of aqueous extracts of pomegranate peel from Italian and local Sardinian varieties and to study their antimicrobial and antibiofilm properties against pathogenic and protechnological microorganisms. Using a multivariate statistical approach, the final objective was to identify the chemical components responsible for the biological action. As the bioactive compound content of the fruit is also related to its growing region, growth stages, pedo-climatic, and ecological conditions (Russo et al., 2018), the environmental, bioclimatic, and agronomic conditions were kept the same for all varieties.

2. Materials and Methods

2.1. Chemicals

The analytical reagents Folin-Ciocalteu reagent, aluminium chloride, 2,2′-azino-bis-(3-ethylbenzothia zoline-6-sulphonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (Milan, Italy). The high-performance liquid chromatography (HPLC) grade standards for punicalin *α* and *β*, punicalagin *α* (Pun*α*) and punicalagin *β* (Pun*β*), hydroxybenzoic acid, catechin, epicatechin, chlorogenic acid, caffeic acid, rutin, ellagic acid, cyanidin 3,5-diglucoside chloride, and delphinidin 3,5-diglucoside chloride were purchased from Sigma-Aldrich (Milan, Italy). The standard for cyanidin 3-O-glucoside was purchased from Extrasynthese (Genay, France). Gallic acid was from Carlo Erba Reagenti SpA (Rodano, Milan, Italy). Ultrapure water was prepared using a Milli-Q system (Millipore Corporation, Billerica, USA).

2.2. Preparation of pomegranate peel extracts

The chemical composition and antimicrobial activities of the peel from seven pomegranate varieties were evaluated. Fruits were harvested on October 20th, 2020, from the following *P. granatum* varieties: Wonderful (WF), Primosole (PS), Mollar de Elche (ME), Sassari 1 (SS1), Sassari 2 (SS2), Sassari 3 (SS3), and Arbara Druci (AD). All trees are located in the pomegranate varietal collection field of the University of Sassari's Experimental Station "A. Milella" in Oristano, Sardinia (San Quirico-Fenosu, 39°54′12″N, 8°37′19″E), situated 13 m above sea level. The pomegranate orchard was planted in 2016 according to a 6.0 m × 4.5 m planting distribution. Trees are pruned annually, and spontaneous vegetation is controlled by mowing. Drip irrigation is activated during the summer (approx. 2 500 m^3 per hectare per year). The bioclimate of the study area is classified as thermo-Mediterranean, with annual mean, maximum and minimum average temperatures of 17.1 °C, 25.4 °C (July), and 9.6 °C (February), respectively. Precipitation is concentrated in the autumn and winter seasons, with mean annual precipitation of 581 mm (data from the Environmental Protection Agency of Sardinia, Fig. S1).

Five pomegranate fruits per genotype were washed with distilled water. The peel and the arils were manually separated, and the peel was chopped into small pieces using a sharp knife, then dried using a vacuum freeze dryer for 72 h at –55 °C. The dried peel was ground into a fine powder using a laboratory blender (Waring Commercial Blender 7011S). The 1.5 g pomegranate powder was extracted with 25 mL ultrapure water with the solvent:sample mass ratio of 15:1) at 20 or 40 °C for 4 h in a thermostatic bath. Then, samples were centrifuged at 5 000 r/min for 10 min, and the filtrate passed through a 0.45 mm hydrophilic nylon membrane. Samples of PPE were stored at –20 °C until the analysis.

2.3. Determination of PPE total phenolic content

The PPE total phenolic content was assessed by Folin-Ciocalteu assay (Deiana et al., 2019). Briefly, aliquots of the diluted samples were mixed in a 25 mL volumetric flask with Folin-Ciocalteu reagent (1:1) and 10 mL sodium carbonate solution 7.5% and incubated for 2 h at room temperature. Total phenolic content was determined by spectrophotometric analysis (8453 UV-Vis Spectrophotometer, Agilent Technologies, USA) as absorbance at 750 nm, and expressed as mg of gallic acid equivalents per gram of freeze-dried matter (mg GAE per g DW) using a gallic acid calibration curve (10–100 mg/L, R^2 = 0.989). Samples were analyzed in triplicate.

2.4. Determination of PPE total flavonoid content

Total flavonoids were quantified by colorimetric assay according to the $AlCl_3$ method and following previously reported procedures (Re et al., 2019). Quantification was carried out using a catechin calibration curve (2.5–20.0 µg/mL, R^2 = 0.996). Results are expressed as mg of catechin equivalent per g of freeze-dried matter (mg CE per g DW).

2.5. Determination of PPE total tannin content

Analysis of condensed tannins was carried out by vanillin assay, as reported by Melito et al (2016). The absorbance of vanillin-tannin adducts was detected spectrophotometrically at 500 nm, and concentrations were calculated using a catechin calibration curve (1–6 µg/mL, R^2 = 0.998). Results are expressed as mg CE per g DW.

2.6. The PPE antioxidant capacity

The antioxidant capacity of pomegranate extract was evaluated using both DPPH and ABTS methodologies according to the procedures reported in Piluzza et al (2020). Briefly, for each assay, 0.1 mL of appropriately diluted PPE was mixed with 3.9 mL of 60 µmol/L DPPH or 7 mmol/L ABTS, and then stored in the dark for 120 or 6 min, respectively. For both assays, a calibration curve was generated for Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (2–12 µmol/L; R^2 = 0.997 for DPPH assay and R^2 = 0.998 for ABTS assay). Spectrophotometric readings (8453 UV-Vis Spectrophotometer, Agilent Technologies, USA) were carried out at 515 nm for DPPH and at 734 nm for ABTS. Results are expressed as mmol of Trolox equivalents per 100 g of dry weight, (mmol TEAC per 100 g DW).

2.7. The HPLC analysis of phenolic compounds

Reverse-phase HPLC analysis of phenolic compounds was performed using a liquid chromatography system (Agilent 1100, Agilent Technologies, Palo Alto, USA) equipped with a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A), and a diode array detector (G1315 B, DAD). Chromatographic separation was achieved with a Luna C18 column (250 mm × 4.6 mm, 5 µm) from Phenomenex (Torrance, USA) with a security guard cartridge (4 mm × 2 mm). The flow rate was set at 1 mL/min, and the column temperature was set to 30 °C. Elution was carried out with a binary mobile phase of solvent A (water and 0.1% trifluoracetic acid) and solvent B (acetonitrile). The gradient elution program was as follows: 0 min, 99% A; 5 min, 95% A; 6 min, 93% A; 10 min, 85% A; 15 min, 75% A; 20 min, 10% A; 25 min, 99% A, with a post-time of 2 min. Detection was performed at 280, 360, and 520 nm. Phenolics were identified according to the retention time of a mixture of standards and quantified using the respective calibration curves. Samples were appropriately diluted before injection. The results are presented as milligrams per gram of dry weight (mg/g DW). To detect anthocyanins, 2 mL pomegranate extract was loaded into C18-Sep-Pak cartridges (Strata C-18-E, 500 mg per 6 mL, Phenomenex) previously conditioned with 2 mL methanol, followed by 5 mL of 5 mmol/L H_2SO_4. After washing with 5 mL of 5 mmol/L H_2SO_4, the anthocyanins were eluted with 5 mL MeOH followed by 5 mL ultrapure water into a 10 mL calibrated flask.

2.8. Antimicrobial activity of PPE

2.8.1. Bacterial strains

The following bacterial strains were used in this study: *Staphyloccocus aureus* DSM 20231, *Staphyloccocus aureus* DSM 2569, *Staphyloccocus aureus* DSM 6148, *Listeria monocytogenes* DSM 20600, *Listeria monocytogenes* DSM 15675, *Salmonella bongori* DSM 13772, *E. coli* DSM 30083 and DSM 4415, *Lacticaseibacillus casei* Shirota, and *Limosilactobacillus reuteri* DSM 17938. All strains, with the exception of the lactic acid bacteria (LAB), were cultivated overnight at 37 °C in brain heart infusion agar (BHI) for the determination of PPE antimicrobial activities and to detect biofilm formation. The LAB were cultivated overnight at 30 °C in De Man, Rogosa, and Sharpe (MRS) agar.

2.8.2 Determination of minimum inhibitory concentration

The antimicrobial activities of PPE were quantitatively evaluated in vitro by measuring the MIC (Petretto et al., 2018). Concentrations ranging from 0.09 to 3.00 mg/mL of each extract (from both room temperature (20 °C) and warm (40 °C) water extractions) were prepared. Subsequently, 100 µL of each concentration were added to wells of a 96-well microtiter plate containing a total volume of 100 µL Müller-Hinton broth concentration 2X (MHB 2X) plus bacterial inoculant of approximately 5×10^5 CFU/mL final concentration of the tested bacteria. Negative control wells contained non-inoculated medium plus PPEs. Positive control wells contained inoculated MHB with no PPE. Plates were then incubated at 37 °C for 24 h. Inhibition of bacterial growth was determined visually, and the MIC was defined as the lowest concentration of the extract that inhibited microorganism growth at the end of the incubation period. All the bacterial strains were tested in triplicate, and the assay was repeated twice for each strain.

2.9. Antibiofilm activity of PPE

The quantitative analysis of the ability of pathogenic bacteria strains to form biofilms was evaluated by crystal violet (CV) assay, according to the methods reported in Xu et al. (2016) with some modifications. Subsequently, only three PPEs (ME, PS, and SS3) were processed further to test the extracts' activities against planktonic bacteria and their ability to block biofilm formation. Concisely, 100 µL of the bacteria cell suspensions containing 5×10^6 cells per mL TSB were transferred to microtiter plates. Then, 100 µL of different concentrations of PPEs, ranging from 0.09 to 3.00 mg/mL, according to the MIC of each strain (the MIC and two sub-MIC concentrations), were added to each well. The bacterial strains tested were selected according to their biofilm formation ability. Negative controls were wells containing non-inoculated medium. Positive controls were wells containing inoculated culture medium but no PPE. The microplates were incubated at 30 °C for 72 h; the CV assay was then completed as per test plates, and the optical density (OD) values were measured spectrophotometrically.

2.10. Statistical analyses

The effect of pomegranate variety on the capacity of pomegranate peel water extracts to limit biofilm formation was assessed by analysis of variance (ANOVA). When a significant effect was observed ($P < 0.05$), the differences between means were separated using the Tukey-Kramer multiple comparisons test. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) models were developed to distinguish the different pomegranate varieties from one another according to the chemical composition of their respective PPEs. A preliminary exploratory projections to latent structures discriminant analysis (PLS-DA) model including seven class levels (i.e., all seven pomegranate varieties) was performed to investigate the distances between varieties. Then, in order to select the most discriminatory variables for each variety with high accuracy, models for each of the seven varieties vs. all others were developed (Deiana et al., 2019). The OPLS analysis was used to perform the pairwise models due to its simpler interpretation with respect to PLS analysis.

Indeed, OPLS concentrates the predictive information in one component, and the information not correlated to *Y* is included in further orthogonal components. Furthermore, PLS-DA models were generated to assess the role of bioactive molecules, both individually and as combinations of molecules, on the antimicrobial activity of PPE. A model was developed for each tested bacterial strain. The antimicrobial activity was expressed according to the results of MIC analyses: MIC values were grouped as "High" (0.09–0.38 mg/mL), "Medium" (0.75–3.00 mg/mL), or "No" (no antimicrobial activity), and these classes were used for the PLS-DA models. In this case, due to the presence of multiple classes in several models and to maintain a uniform method of analysis, only PLS-DA models were performed. All variables were included in the models at the start, then those with the lowest model relevance, according to the variable influence on projection (VIP) values, were gradually excluded until a statistically significant model was produced according to its performance parameters R^2Y and Q^2, and the permutation tests used to validate the models (Deiana et al., 2022). The parameter R^2Y indicates the percentage of variation explained by the model, whereas Q^2 indicates the proportion of variance predictable by the model. Finally, two OPLS regression models, setting the *Y* variable as DPPH and ABTS, respectively, were performed to identify the pomegranate components with the highest antioxidant activities. R-Studio software (R version 4.1.1, 2021-08-10, ropls package from Bioconductor for OPLS, OPLS-DA and PLS-DA), was used to conduct the statistical analyses.

3. Results

3.1. Chemical composition of PPE and varietal models

Table 1 summarizes the content of PPE obtained at two different temperatures (20 and 40 ℃) for seven different cultivars in terms of polar phenols, flavonoids, condensed tannins, and anthocyanins. The data show wide content variability between cultivars, while extraction temperature had only a limited effect on phenolic content. The total phenols (T.Phen) varied from 92.1 mg/g for cultivar AD extracted at 20 ℃ to 150.7 mg/g for cultivar PS extracted at 40 ℃. The PS, together with ME, contained the highest concentration of total flavonoids (T.Flav, 22.2 mg/g in PS; 22.5 mg/g in ME), whereas SS3 and AD contained the lowest (14.3 and 15.4 mg/g, respectively). The values of condensed tannins ranged from 1.6 mg/g (SS2) to 7.9 mg/g (ME).

Table 1 Chemical composition and antioxidant activity of pomegranate peel extracts from seven varieties obtained through cold (20 ℃) and hot (40 ℃) extraction

Metabolite	*T* (℃)	Arbar a Druci	Mollar de Elche	Primosole	Sassari 1	Sassari 2	Sassari 3	Wonderful
GA (mg/g)	20	1.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.9 ± 0.1
	40	0.9 ± 0.2	0.2 ± 0.0	1.0 ± 0.2	0.0 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.9 ± 0.0
Unknown (mg/g)	20	63.5 ± 5.3	8.9 ± 0.6	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.0	2.4 ± 0.0	143.9 ± 9.7
	40	118.1 ± 8.6	13.5 ± 1.0	1.5 ± 0.0	1.3 ± 0.0	0.8 ± 0.1	2.6 ± 0.1	190.0 ± 3.8
Punicalin α (mg/g)	20	13.8 ± 0.3	9.4 ± 0.3	5.9 ± 0.1	4.5 ± 0.0	5.7 ± 0.3	5.2 ± 0.1	29.6 ± 2.5
	40	17.1 ± 0.7	7.3 ± 0.2	7.9 ± 0.5	3.9 ± 1.1	5.9 ± 0.6	5.9 ± 0.0	26.9 ± 0.0
Punicalin β (mg/g)	20	17.1 ± 0.4	6.5 ± 0.3	5.3 ± 0.3	3.0 ± 0.1	3.5 ± 0.1	4.2 ± 0.1	28.8 ± 1.6
	40	23.2 ± 2.0	7.3 ± 0.2	5.4 ± 0.0	4.6 ± 0.2	3.6 ± 0.5	4.5 ± 0.0	40.5 ± 0.5
Punα (mg/g)	20	61.5 ± 5.3	97.2 ± 1.0	101.8 ± 0.6	80.4 ± 1.1	84.4 ± 0.4	58.9 ± 2.8	38.0 ± 0.3
	40	27.3 ± 2.2	120.9 ± 0.3	125.3 ± 1.9	85.1 ± 1.6	88.7 ± 3.7	84.9 ± 1.1	17.6 ± 3.0
4-HbA (mg/g)	20	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
	40	0.00 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
Punβ (mg/g)	20	110.8 ± 6.7	205.3 ± 3.1	201.4 ± 4.0	156.7 ± 0.4	174.3 ± 3.0	177.8 ± 9.3	81.3 ± 2.5
	40	51.8 ± 5.2	241.3 ± 2.3	258.6 ± 6.1	167.2 ± 8.4	186.7 ± 7.9	179.0 ± 4.0	45.0 ± 4.8
Cat (mg/g)	20	3.5 ± 0.0	3.9 ± 0.2	3.8 ± 0.0	3.4 ± 0.1	4.7 ± 0.1	3.1 ± 0.0	3.9 ± 1.4

ChlA (mg/g)	40	3.4 ± 0.0	4.5 ± 0.2	5.5 ± 1.0	3.4 ± 0.1	5.7 ± 0.2	3.5 ± 0.1	2.9 ± 0.1
	20	1.1 ± 0.0	1.2 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	0.8 ± 0.0	1.5 ± 0.0	1.2 ± 0.1
CA (mg/g)	40	1.1 ± 0.0	1.4 ± 0.0	1.6 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	1.7 ± 0.1	1.1 ± 0.1
	20	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
EpiCat (mg/g)	40	0.2 ± 0.0	0.4 ± 0.0	0.9 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	0.4 ± 0.0	0.5 ± 0.0
	20	0.6 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.0
Rut (mg/g)	40	0.6 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.7 ± 0.0
	20	0.4 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.4 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.7 ± 0.0
EA (mg/g)	40	0.4 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	0.4 ± 0.0	0.9 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
	20	12.6 ± 0.0	9.8 ± 1.0	16.8 ± 0.6	19.0 ± 0.8	16.0 ± 0.1	15.6 ± 0.4	28.7 ± 1.5
Cya-3-glu (mg per 100 g)	40	16.6 ± 2.1	10.6 ± 0.2	17.9 ± 1.1	20.7 ± 1.2	18.0 ± 1.0	18.6 ± 0.1	30.8 ± 1.6
	20	2.3 ± 0.0	0.0 ± 0.0	2.2 ± 0.0	3.4 ± 0.2	0.0 ± 0.0	2.7 ± 0.0	3.3 ± 0.1
Del-3-glu (mg per 100 g)	40	2.4 ± 0.0	0.0 ± 0.0	2.3 ± 0.0	3.5 ± 0.0	0.0 ± 0.0	2.8 ± 0.0	3.2 ± 0.1
	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2 ± 0.0	2.7 ± 0.1
Pel-3-glu (mg per 100 g)	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.0	0.0 ± 0.0	2 ± 0.0	2.6 ± 0.0
	20	2.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	2.1 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	2.3 ± 0.1
Cya-3,5-diglu (mg per 100 g)	40	2.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	2.2 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	2.3 ± 0.0
	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.0
Del-3,5-diglu (mg per 100 g)	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.0
	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0
Pel-3,5-diglu (mg per 100 g)	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0
	20	2.1 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	2.9 ± 0.1	0.0 ± 0.0	2.6 ± 0.1	4.0 ± 0.1
DPPH (mmol per 100 g)	40	2.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.0	3.2 ± 0.1	0.0 ± 0.0	2.7 ± 0.0	3.9 ± 0.0
	20	48.2 ± 1.4	52.0 ± 1.3	58.0 ± 0.7	46.8 ± 1.9	52.4 ± 3.9	43.0 ± 1.8	58.6 ± 0.8
ABTS (mmol per 100 g)	40	48.7 ± 1.3	60.3 ± 5.5	68.0 ± 0.3	53.4 ± 2.4	51.9 ± 1.7	45.4 ± 2.0	55.9 ± 1.7
	20	53.6 ± 1.1	68.3 ± 1.1	69.7 ± 2.7	63.4 ± 2.0	63.9 ± 2.3	59.0 ± 0.1	75.7 ± 1.2
T.Flav (mg/g)	40	59.7 ± 0.4	82.8 ± 1.5	87.3 ± 0.4	73.7 ± 1.0	72.6 ± 4.5	66.1 ± 2.1	74.5 ± 3.1
	20	15.4 ± 1.0	20.8 ± 0.5	20.3 ± 0.2	15.5 ± 0.4	16.9 ± 1.6	14.8 ± 0.5	19.3 ± 1.5
T.Tan (mg/g)	40	16.0 ± 0.7	22.5 ± 0.0	22.2 ± 0.7	16.4 ± 0.4	18.0 ± 1.1	14.3 ± 0.6	19.1 ± 1.6
	20	5.1 ± 0.1	7.5 ± 0.2	5.2 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	1.8 ± 0.1	4.2 ± 0.0
T.Phen (mg/g)	40	5.0 ± 0.0	7.9 ± 0.1	5.2 ± 0.2	1.9 ± 0.2	2.3 ± 0.3	1.8 ± 0.1	4.4 ± 0.0
	20	92.1 ± 3.4	127.0 ± 3.3	127.0 ± 1.4	106.0 ± 0.3	126.0 ± 3.4	105.0 ± 1.0	121.4 ± 4.5
	40	93.0 ± 1.5	149.6 ± 1.3	150.7 ± 2.3	121.4 ± 4.3	137.3 ± 6.2	125.9 ± 2.5	111.3 ± 0.4

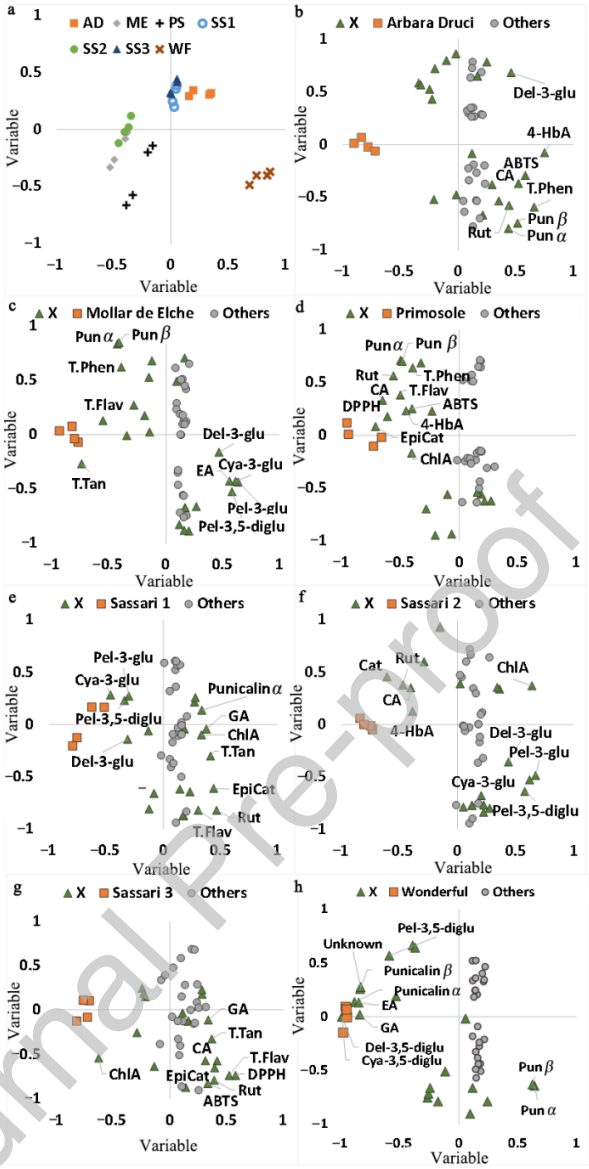
Notes: *T*, temperature; GA, gallic acid; Pun*α*, punicalagin *α*; 4-HbA, 4-Hydroxybenzoic acid; Pun*β*, punicalagin *β*; Cat, catechin; ChlA, chlorogenic acid; CA, caffeic acid; EpiCat, epicatechin; Rut, rutin; EA, ellagic acid; Cya-3-glu, cyaninin-3-glucoside; Del-3-glu, delphinidin-3-glucosid; Pel-3-glu, pelargonidin-3-glucoside; Cya-3,5-diglu, cyanidin-3,5-diglucoside; Del-3,5-diglu, delphinidin-3,5-diglucosid; Pel-3,5-diglu, pelargonidin-3,5-diglucoside; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis-(3-ethylbenzothia zoline-6-sulphonic acid); T.Flav, total flavonoids; T.Tan, total tannins; T.Phen, total phenols.

Eighteen phenolic compounds were identified by HPLC-DAD: six hydrolysable tannins (punicalin *α* and punicalin *β*, Pun*α* and Pun*β*, ellagic acid, and gallic acid), two phenolic acids (4-hydroxybenzoic acid, caffeic acid), chlorogenic acid, two flavan-3-ols (catechin and epicatechin), one flavonoid glycoside (rutin), and six anthocyanins. The level of each phenolic compound in the peel was mostly cultivar dependent and related to the extraction temperature to a lesser extent. The ellagitannins were the predominant components; with the two punicalagin anomeric structures, Pun*α* and Pun*β*, being the most abundant phenolics (17.6–125.3 mg/g and 45.0–258.6 mg/g, respectively). Cultivars ME and PS had the highest concentrations of punicalagin in their peel extracts, while WF had the lowest. Both the *α* and *β* anomers of punicalin were detected (3.9–29.6 mg/g and 3.0–40.5 mg/g, respectively), with the highest concentrations found in cultivars WF and AD. An unknown peak of great intensity was detected in cultivars WF and AD (190.0 and 118.1 mg/g, respectively). According to the literature data, this peak is sometimes recognized as punicalin (Zhang et al., 2009; Živković et al., 2018) or as an unspecified ellagitannin (Romani et al., 2012). In this work, we recognized the punicalin *α* and punicalin *β* peaks based on the retention times for the pure standards. Therefore, we decided to consider the unknown peak as an ellagitannin, to quantify it as an ellagic acid derivative, and to label the peak as "unknown". Cultivar WF contained the highest concentration of ellagic acid (EA, 28.7–30.8 mg/g), whereas the other six cultivars consistently reported lower values (9.8–20.7 mg/g). All other compounds detected were at levels below 5.5 mg/g.

Six major anthocyanin compounds were identified in pomegranate peel extract, namely delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, delphinidin-3-glucoside, cyanidin-3-

glucoside, and pelargonidin-3-glucoside, with large differences in concentration between cultivars. None of these molecules were present in cultivars ME and SS2. Delphinidin-3,5-diglucoside and cyanidin-3,5-diglucoside were only observed in WF peel extracts, which showed the highest concentrations for all the anthocyanins detected.

In order to investigate differences between cultivars, a supervised PLS-DA and OPLS-DA multivariate approach was adopted. According to the preliminary "7-cultivars" PLS-DA model based on the respective peel extract compositions, PS and WF were identified as the varieties that differed from the others the most (Fig. 1a). The remaining varieties clustered into two groups: the first comprised SS2 and ME, probably due to the common absence of anthocyanins, whereas the second consisted of SS1, SS3, and AD. To highlight the varietal-specific features of PPE further, models were generated investigating "single variety vs all others" (Figs. 1b–1h) which achieved both good fit (R^2Y > 0.90, Table S1) and predictability (Q^2Y > 0.88). Specifically, AD, SS1, and SS3 stood out for their overall low concentration of phenols, tannins, and flavonoids, with the exception of chlorogenic acid in the case of SS3, and anthocyanins in SS1. On the other hand, SS2 and ME were distinguished by their absence of anthocyanins and good presence of flavonoids and phenolic acids, specifically catechin, rutin, caffeic acid, and 4-hydroxybenzoic acid. The ME and PS varieties presented the highest concentrations of punicalagin isomers. The PS was also characterized as producing the peel with the strongest overall antioxidant activity due to its high concentrations of epicatechin, caffeic acid, rutin, Punα, Punβ, chlorogenic acid, and 4-hydroxybenzoic acid. Finally, WF extracts were distinguished by their high concentrations of anthocyanins, ellagic acid, and punicalin.



WF: Wonderful; PS: Primosole; ME: Mollar de Elche ; SS1: Sassari 1; SS2: Sassari 2; SS3: Sassari 3; AD: Arbara Druci.

Fig. 1 Score plot of projections to latent structures discriminant analysis (PLS-DA) (a, 7-cultivars model) and correlation biplots for orthogonal projections to latent structures discriminant analysis (OPLS-DA) models (b−h) showing both relationships between scores (observations classified by varieties: b, Arbara Druci, c, Mollar de Elche; d, Primosole; e, Sassari 1; f, Sassari 2; g, Sassari 3; h, Wonderful) and loadings (*X* variables: pomegranate peel extract (PPE) chemical composition). Observations close to *X* variables have high concentrations of such variables and low concentrations in variables situated opposite. Labelled variables are those with predictive variable influence on projection (VIP) values above 1.

3.2. Antioxidant activity of PPE

The antioxidant activities of PPEs obtained at the two different extraction temperatures were generally similar. The largest differences in ABTS values were observed for PPEs from ME and PS, being 82.8 and 87.3 mmol per 100 g, respectively, for hot water (40 °C) extraction versus 68.3 and 69.7 mmol per 100 g, respectively, for cold water (20 °C) extraction (Table 1). The differences in values obtained between the two extraction

temperatures were even lower for DPPH values, with a maximum difference of 10 units between the two water temperatures. As for ABTS, ME and PS were the two varieties demonstrating the greatest differences, with hot water extraction resulting in higher phenol concentrations. The AD and SS3 were the varieties which showed the lowest overall antioxidant activities. According to the OPLS models (Fig. 2), total flavonoids, specifically epicatechin and rutin, where the chemical compounds with the highest antioxidant activity. Moreover, the level of phenols such as caffeic acid and 4-hydroxybenzoic acid in PPE strongly correlated with the DPPH and ABTS values. On the other hand, tannins, such as the punicalagins, contributed only marginally to the antioxidant activity of PPE, whilst anthocyanins, punicalin, and ellagic acid did not make a significant contribution.

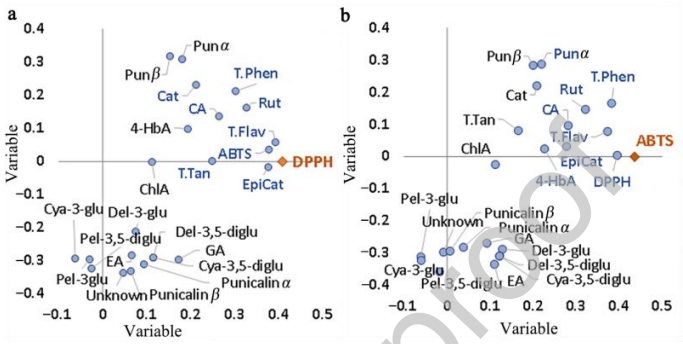


Fig. 2 The OPLS loading scatter plots representing relationships between *X* variables (PPE chemical composition) and *Y* variable: 2,2-diphenyl-1-picrylhydrazyl (DPPH) (a) and 2,2′-azino-bis-(3-ethylbenzothia zoline-6-sulphonic acid) (ABTS) (b). The variables highlighted in bold blue type are those strongly correlated with *Y* variable (in terms of variable importance on projection: VIP).

3.3. Antibacterial activity of PPE

The antibacterial activities of the PPEs from the different cultivars were assessed by determining the MIC for each bacteria strain tested (Table 2). Most of the PPEs showed some effectiveness at suppressing microbial growth, with a MIC of the seven extracts from cold and hot water extraction ranging from 0.09 to 3.00 mg/mL, and Gram-negative bacteria notably more resistant than Gram-positive bacteria. A comparison of the antimicrobial activity of all PPEs showed that the most efficacious were those obtained from ME, PS, and SS3; WF extract showed the weakest antimicrobial activity. The *S. aureus* and *L. monocytogenes* showed high susceptibility to nearly all the PPEs tested. The *La. casei* Shirota and *Li. reuteri* DSM 17938 showed less susceptibility to the PPEs compared with Gram-positive bacteria. In contrast, *Sa. bongori* DSM 13772 showed resistance to all the PPEs tested, whereas the *E. coli* strains showed resistance against most of the PPEs.

Table 2 Minimal inhibitory concentration (mg/mL) of pomegranate peel extracts obtained through cold and hot water extraction against ten bacteria strains

Metabolite	*T* (°C)	Arbara Druci	Mollar de Elche	Primosole	Sassari 1	Sassari 2	Sassari 3	Wonderful
S. aureus DSM 20231	20	3.00	0.75	0.75	ni	0.75	0.75	ni
	40	3.00	3.00	0.75	0.75	ni	1.50	ni
S. aureus DSM 2569	20	1.50	0.38	0.75	0.75	0.75	0.75	ni
	40	1.50	0.38	0.38	0.75	ni	ni	1.50
S. aureus DSM 6148	20	1.50	0.75	0.75	ni	0.75	0.75	ni
	40	ni	0.75	1.50	ni	0.75	1.50	ni
L. monocytogenes DSM 15675	20	0.19	3.00	0.09	ni	0.09	0.19	ni
	40	0.09	1.50	0.75	ni	ni	0.75	ni
L. monocytogenes DSM 20600	20	ni	0.75	ni	ni	ni	0.75	ni
	40	ni	0.38	ni	ni	ni	1.50	ni

E. coli DSM 4415	20	ni	1.50	3.00	ni	3.00	3.00	ni
	40	3	3.00	ni	ni	3.00	ni	ni
E. coli DSM 30083	20	ni	ni	ni	ni	ni	ni	ni
	40	ni	ni	ni	ni	ni	0.75	1.50
Sa. bongori DSM 13772	20	ni	ni	ni	ni	ni	ni	ni
	40	ni	ni	ni	ni	ni	ni	ni
La. casei Shirota Yacult	20	ni	1.50	ni	ni	ni	ni	ni
	40	ni	1.50	3.00	ni	ni	ni	ni
Li. reuteri DSM 17938	20	3.00	1.50	0.75	3.00	1.50	3.00	ni
	40	3.00	1.50	0.75	3.00	3.00	3.00	3.00

Notes: ni, no inhibition; *T*, temperature. *S.*, *Staphyloccocus*; *L.*, *Listeria*; *Sa*, *Salmonella*; *E.*, *Escherichia*; *La.*, *Lacticaseibacillus*; *Li.*, *Limosilactobacillus*.

The PLS-DA analysis enabled us to identify the bioactive compounds contributing the most to the antimicrobial activities of PPE. Table 3 reports the molecules associated with either "Medium" or "High" antimicrobial activity (according to the highest VIP values) for each microbial strain. Eight PLS-DA models reported significant values of R^2Y and Q^2Y (see also Table S1 and Fig. S2 for model performance parameters and validation analyses, and S4 for relative correlation biplots showing the relationships between *X* and *Y* loadings). No significant model was obtained for *E. coli* DSM 30023 or *Sa. bongori* DSM 13772. Indeed, a "Medium" level of antimicrobial activity against *E. coli* DSM 30023 was observed for SS3 (MIC = 0.75 mg/mL) and WF (1.5 mg/mL) extracts only. On the other hand, *E. coli* DSM 4415 was sensitive to a greater number of the PPE tested, suggesting a possible inhibitory role of Pun*α* and Pun*β*, rutin, and catechin. It is worth noting that the two ellagitannin isomers exhibited good activity against all the bacterial species tested. Other relevant species-specific and strain-specific antimicrobial activities could be attributed to total and individual flavonoids. Specifically, epicatechin was relevant against all *L. monocytogenes* and *S. aureus* strains tested, *Li. reuteri* DSM 17938, and *La. casei* Shirota, whereas catechin contributed to the inhibition of *E. coli* DSM 4415 and *S. aureus* DSM 6148. Chlorogenic acid exhibited high antimicrobial activity against *L. monocytogenes* and *S. aureus* strains. Finally, high values of total tannins and total phenols were positively correlated with the PPE antimicrobial activity against most of the strains tested.

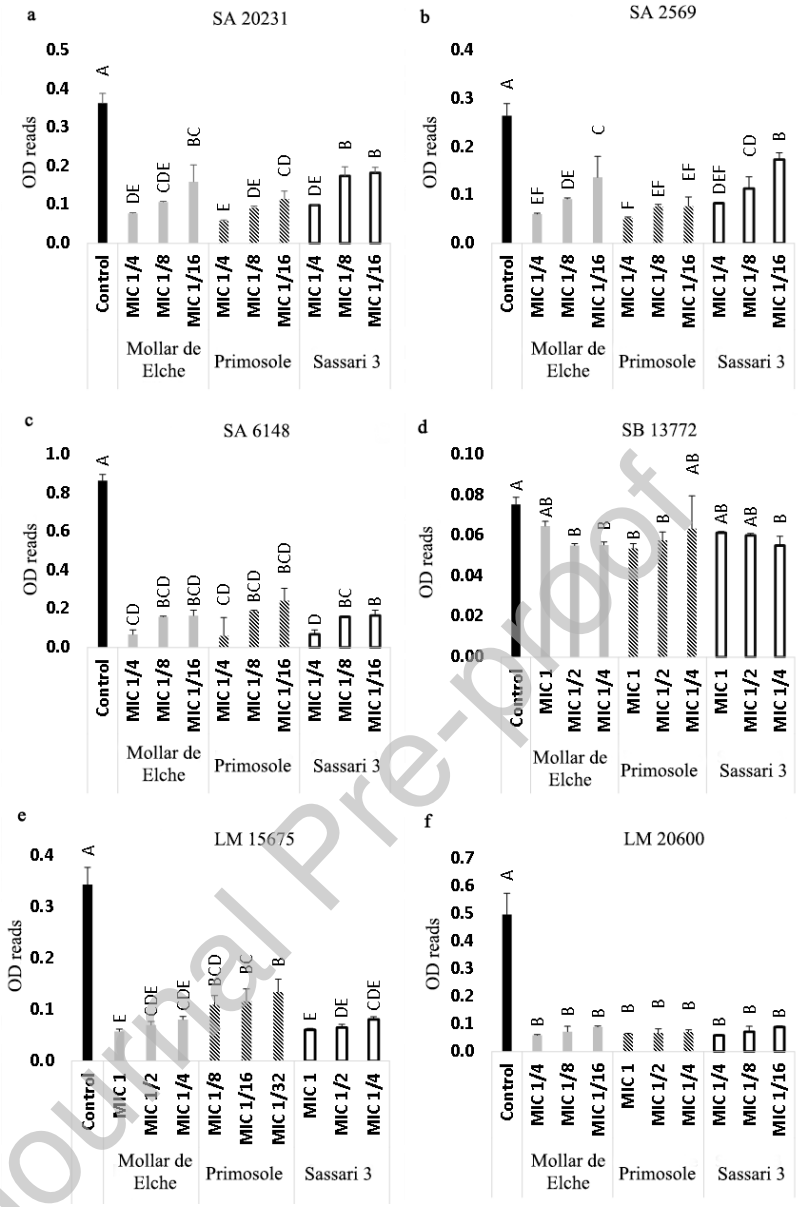
Table 3 A list of variables (phenolic compounds, tannins, flavonoids, and antioxidant activity) mostly related to antimicrobial activity of pomegranate peel extract (PPE) according to projections to latent structures discriminant analysis (PLS-DA) models performed per each bacterial strain tested

Strain	Variable
S. aureus 20231	Chlorogenic acid, total tannins, Pun*β*, Pun*α*
S. aureus 2569	Total tannins, total phenols, epicatechin, total flavonoids
S. aureus 6148	Pun*β*, Pun*α*, catechin, total flavonoids, chlorogenic acid
L. monocytogenes 15675	Chlorogenic acid, total phenols, epicatechin, Pun*α*, total tannins, Pun*β*
L. monocytogenes 20600	Total tannins, total flavonoids, chlorogenic acid, Pun*β*, epicatechin, Pun*α*
E. coli 4415	Pun*α*, Pun*β*, total tannins, rutin, catechin
La. casei Shirota	Total tannins, epicatechin, total flavonoids, total phenols, Pun*α*, Pun*β*, rutin
Li. reuteri 17938	Epicatechin, total flavonoids, DPPH, caffeic acid, Pun*α*, Pun*β*

Notes: In the PLS-DA models, antimicrobial activity was classified according to MIC values reported in Table 2 as "High" (MIC = 0.094–0.375 mg/mL), "Medium" (0.75–3.00 mg/mL), or "No" (no antimicrobial activity).

3.4. Antibiofilm activity of PPE

Among the bacterial strains tested, *S. aureus* and *L. monocytogenes* showed the highest biofilm formation abilities (strong biofilm producers), whereas *Sa. bongori* and *E. coli* strains showed the least biofilm formation ability (weak biofilm producers). To test the capacity of PPEs to inhibit biofilm formation, CV assays were performed using three different concentrations of cold water extracts (20 °C) from three selected cultivars, for which the MIC values ranged from 3 to 0.09 mg/mL (Fig. 3). The PS extract reported the strongest inhibitory activity, reducing the biofilm development of *S. aureus* strains by more than 65% at a sub-MIC (1/16) concentration of 0.19 mg/mL (Fig. 3a–3c). Among the two factors considered in the trial-cultivar and MIC concentration, the latter was predominant, accounting for about 50% of the variance in antibiofilm activity against *S. aureus* strains (Table S2, factorial analysis). In this case, the effect of PPE cultivar provenience was significant for the first two *S. aureus* strains, explaining 35% of the model variance. The *L. monocytogenes* strains (Fig. 3e–3f) also exhibited high sensitivity to the PPEs for which more than 70% reduction of biofilm development was observed, once again at a sub-MIC concentration: 0.019 mg/mL. Finally, *Sa. bongori* DSM 13772 (Fig. 3d) showed the highest resistance to all PPEs tested, and its biofilm formation ability was not significantly affected by sub-lethal concentrations of all PPEs tests.



OD, optical density; SA 20231, *Staphyloccocus aureus* 20231; SA 2569, *Staphyloccocus aureus* 2569; SA 26148, *Staphyloccocus aureus* 6148; SB13772, *Salmonella bongori* 13772; LM 15675, *Listeria monocytogenes* 15675; LM 20600, *Listeria monocytogenes* 20600.

Fig. 3 Effect of three cold water PPE (from Mollar de Elche, Primosole, Sassari 3 cultivars), applied at different concentrations (minimum inhibitory concentrations, MIC), on biofilm formation. The letters above the columns indicate statistical differences according to Tukey's test (MIC 1 = 3 mg/mL; MIC 1/2 = 1.5 mg/mL; MIC 1/4 = 0.75 mg/mL; MIC 1/8 = 0.375 mg/mL; MIC 1/16 = 0.188 mg/mL; MIC 1/32 = 0.094 mg/mL)

4. Discussion

4.1. Varietal composition and antioxidant activity

In agreement with Wang et al. (2011), we found extraction with water at 40 ℃ for 4 h to be an efficient method for the extraction of pomegranate peel antioxidants (phenolics, proanthocyanins, and flavonoids).

However, our data also showed that extraction at this higher temperature was not always accompanied by a higher concentration of extracted compounds compared with extraction at room temperature (20 ℃). Kharchoufi et al. (2018), on the other hand, showed that a further increase in temperature from 40 to 55 ℃ had a significant effect on the extraction of phenolic compounds from the peel of the Gabsi variety of pomegranate. Considering the significant impact in terms of cost for large scale and industrial applications, we did not take higher extraction temperatures into consideration.

The levels of total phenols, flavonoids, and tannins are in line with the previous literature. Saad et al. (2012) reported a total phenolic content of 134.3–181.0 mg/g in pomegranate peels from Tunisian varieties extracted using a methanol：water (80:20) solution. Turrini et al. (2020) found a total phenolic content of 148 mg GAE per g in the Wonderful cultivar, which was slightly higher than our results, obtained through the decoction of peels in ultrapure water. Similarly, Young et al. (2017) reported a total phenol content equal to 134.16 mg GAE per g in peel from Wonderful pomegranates grown in California and extracted with aqueous methanol. Some pomegranate peels from Turkish varieties described by Çam and Hışil (2010) reported total flavonoid and tannin concentrations similar to our findings. By contrast, other varieties from Algeria (Kennas and Amellal-Chibane, 2019) showed higher total phenol and tannin concentrations, but the water extraction process carried out involved longer extraction times. Montefusco et al. (2021) analyzed four different Israeli pomegranate cultivars grown in Southern Italy, and the reported total flavonoids (4.0–5.1 mg CE per g) and TEAC values (ABTS 34.5–41.4 mmol per 100 g DW) were very similar to ours.

El-Beltagi et al. (2022) reported a total phenolic content of 513.8 mg of gallic acid per 100 g in water extracts of pomegranate peel, a much lower value than that obtained in the present study. However, the fruits used in their study had been purchased from a supermarket, thus the storage and shelf life conditions of the fruits were unknown. Conversely Derakhshan et al. (2018) reported a value for the total phenolic content of Iranian pomegranate peel that was two to three-fold that obtained here, but they used a different solvent for extraction and a longer extraction time.

In agreement with our findings, Young et al. (2017) reported Wonderful peel to be poor in punicalagin. Balli et al. (2020) extracted pomegranate peel bioactive molecules from Mollar de Elche and Wonderful varieties through decoction and obtained much lower concentrations of punicalagin isomers in Mollar de Elche compared with our data, but their values for the Wonderful variety were comparable. Gullón et al. (2020) highlighted strong varietal differences in terms of total amounts of hydrolysable tannins. The authors reported a wide range in punicalagin values (98.02–612.80 mg/g) for pomegranate peel methanol: water extracts obtained from Egyptian and Israeli cultivars. Rosas-Burgos et al. (2017), using methanol as extraction solvent, reported ellagic acid concentrations in Spanish pomegranate cultivars, such as Mollar de Elche, that were very similar to those reported here.

Our results are consistent with those found by Gigliobianco et al. (2022), who detected extremely high levels of punicalagin A and B in peel extract of Wonderful and Mollar de Elche pomegranate cultivars grown in the Marche region of Italy. Moreover, as in the present work, they demonstrated higher levels of ellagic acid in peel from the Wonderful variety than from the peel of any other pomegranate variety.

The observed absence of anthocyanins in the peel from the Mollar de Elche variety has also been reported by other authors (Da Silva Veloso et al., 2020, Gigliobianco et al., 2022), as has the high anthocyanin concentration we observed in Wonderful peel (Zhao et al., 2013, Gigliobianco et al., 2022).

Previous studies have attributed the main antioxidant activity of PPE to punicalagins, punicalins, and ellagic acids (Rosas-Burgos et al., 2017; Kumar and Neeraj, 2018). In contrast, our findings attributed a negligible role to the latter two compounds, and only a secondary role to punicalagins. Instead, our results indicated

overall antioxidant activity as partially related to the presence of flavonoids epicatechin, catechim, and rutin, and principally related to the levels of total flavonoids and total phenols. These results suggested that the antioxidant power of a vegetal matter should be primarily ascribed to the specific mixture of its bioactive molecules and their synergic activity. Moreover, the differences between our data and previous literature might be due to the different number of the considered bioactive compounds and to the different applied methods of statistical analysis.

In general, the differences in our quantitative data compared with those in the literature could also be explained by genetic factors, different ripening stages, or pedoclimatic conditions. All these factors can strongly influence the levels and detection of bioactive compounds in pomegranate peel, making it difficult to compare published works. In our experiment, we could conclude that a significant genotype-dependent variability in content was observed since environmental, agronomic, and analytical conditions were the same for all varieties. Moreover, it is worth noting that, to the best of our knowledge, this study is the first to describe in detail the PPE composition of Primosole, an Italian pomegranate cultivar of international commercial interest, and the Sardinian varieties Arbara Druci, Sassari 1, Sassari 2, and Sassari 3.

With regard to the Sardinian varieties, the results reported in this study highlight the importance of preserving and exploiting local varieties as a source of nutraceutical compounds, which provided producers with more selection possibilities in accordance with the market's demands or production goals. In the case of the four Sardinian varieties investigated, it is worth noting the good punicalagin concentrations, close to the highest values observed in Mollar de Elche and Primosole, in PPEs from Sassari 1, Sassari 2, and Sassari 3. Sassari 1 and Sassari 2 were also good sources of ellagic acid, catechin, and cyanidin-3-glucoside, whereas Arbara Druci can be considered a good source of punicalins and total tannins.

4.2. Antimicrobial and antibiofilm activity

Naturally-derived antimicrobials from various plant sources, including *P. granatum*, have been successfully applied as alternatives to synthetic chemicals for suppressing the growth of a number of foodborne bacteria (Kanatt et al., 2010). The antimicrobial properties of plants can probably be attributed to their specific secondary metabolites (Bensilmane et al., 2020; Balaban et al., 2021), which vary due to the variance in the chemical composition and as a result the mechanism of action from one plant extract to another (Hanafy et al., 2021). Noteworthily that *P. granatum* peel extracts antimicrobial activity was reported in several previous studies against different foodborne pathogens. These studies provide evidence for the presence of polyphenolic bioactive compounds in PPE that was effective against the growth of several microbes including *S. aureus*, *E. coli*, *L. monocytogenes*, and *S. enteritidis*, *Aspergillus niger*, *Saccharomyces cerevisiae* (Malviya et al., 2014; Assar and Shahate, 2017; Benslimane et al., 2020; Balaban et al., 2021), *Fusarium sambucinum*, *Penicillium italicum*, *Bacillus subtilis* (Chen et al., 2020), *S. epidermidis*, *Klebsiella pneumoniae*, *S. typhi*, *Yersinia enterocolitica*, and *Candida albicans* (Qahir et al., 2021).

In the current study, we screened the antimicrobial and antibiofilm activities of different locally cultivated (Sardinia) pomegranate cultivars against several food pathogens and probiotic bacteria. The initial screening for antimicrobial activity showed that most of the PPEs were effective in suppressing the microbial growth of the tested bacteria, albeit to different degrees. We also found that the Gram-positive strains *S. aureus* and *L. monocytogenes* were more sensitive than *E. coli* and *Sa. bongori* (Gram-negative) strains (Table 2) to the tested extracts. In addition, the MIC values recorded in the present study (0.09 to 3.00 mg/mL) were much lower than those reported in previous studies (Wafa et al., 2017; Nasreddine et al., 2018; Nozohour et al., 2018). Hanafy et al. (2021) reported in a study to evaluate the antimicrobial potentials of different fruit peels that PPEs showed the most significant inhibitory effect against the tested strains *B. cereus*, *S. aureus* MRSA, *S.*

aureus, *L. monocytogenes*, and *S. typhimurium* where the MIC values ranged between 6.25 and 12.5 mg/mL. Pagliarulo et al. (2016) showed that pomegranate Phyto-complex extracts had an effective inhibitory effect on the bacterial growth of clinical isolates of *S. aureus* and *E. coli* with a MIC ranging from 20 to 30 mg/mL. Moreover, in several studies, the MIC values were observed to be 0.62–10.00 mg/mL against *S. aureus*, *E. coli*, and *P. aeruginosa*. Such antimicrobial variances could be a result of the different extraction methods and different used solvents, fruit freshness, time, and region of cultivation (Hany et al., 2011; El-Beltagi et al., 2022).

Among the strains investigated by Rahnemoon et al. (2016), the authors reported *S. aureus*, followed by *L. monocytogenes*, showed the highest sensitivity to PPEs, whereas *E. coli* showed the lowest sensitivity. This is in line with the results of the current study, with the addition of *Sa. bongori* which showed complete resistance to all the tested PPEs. Inconsistently, different levels of inhibition for the different studied pomegranate peel extracts were reported, where the *Salmonella* strain was the most sensitive among the tested pathogens (Abou El-Nour, 2019). This might be due to a strain-specific action of PPE, which could be more or less efficient against strains of the same species.

Gram-positive bacteria were reported to be more sensitive to PPEs than Gram-negative bacterial strains, which is consistent with the results obtained in this work. This variation in sensitivity could be ascribed to differences in cell wall composition (Kanatt et al., 2010; Alexandre et al., 2019; Hanani et al., 2019; Benslimane et al., 2020). Gram-positive bacteria lack an outer membrane which contributes to the easier diffusion of phytochemicals through the cell wall. The outer membrane of Gram-negative bacteria has a lipopolysaccharide layer and periplasmic space in the cell wall that hinders the penetration of antimicrobial substances. However, in Gram-positive bacteria, the plant extracts are able to disrupt the molecular structure of the bacterial cell wall, reacting in several ways and ending with cell death, which may explain the different resistances of Gram-positive and Gram-negative bacteria to the actions of phenolic compounds (Al-Zoreky, 2009; Alexandre et al., 2019; Benslimane et al., 2020; Balaban et al., 2022).

Phenolic compounds can react through different mechanisms of action to express their antimicrobial activity against microbes. This can be achieved by inhibiting several virulence factors (e.g., by inhibition of biofilm formation, neutralization of bacterial toxins, and reduction of host ligand adhesion), reducing membrane fluidity, and inhibiting the synthesis of nucleic acids or energy metabolism (Takó et al., 2020). One potential mechanism to explain the antimicrobial activity of phenolic compounds against foodborne pathogens could be the hyperacidification of the plasma membrane interphase resulting from phenolic acid dissociation. This procedure leads to changes in the cell's membrane potential which would increase its permeability. This mechanism may also explain the differences in the sensitivity of the different pathogenic microorganisms toward phenolic acids (Alexandre et al., 2019).

Punicalagin is one of the main ellagitannins (ETs) present in pomegranate peel extract that is found to be responsible for its antimicrobial potentials where it was observed to inhibit the growth of several bacterial strains, such as *S. aureus*, *S. epidermidis*, *S. xylosus*, *S. enteritidis*, *E. coli.*, *C. albicans*, *Pseudomonas aeruginosa*, *B. cereus*, *Lactobacillus sakei* ssp. Sakei, *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Enterococcus* spp. (Maphetu et al., 2022). In addition, Rosas-Burgos et al. (2017) suggested punicalagin and ellagic acid be the bioactive molecules principally responsible for the antimicrobial activity of pomegranate peel. This is partially in line with the results of the current study, where the inhibitory effect was primarily correlated with Pun*α* and Pun*β*, but also with other flavonoid and phenolic molecules. However, the PLS-DA models investigating the relationship between PPE composition and relative antimicrobial activity only partially describe the biological process at play. Indeed, the presence of other broad-spectrum antimicrobial

compounds, such as organic acids, could also contribute to the antibacterial activity of PPEs (Devatkal et al., 2013; Malviya et al., 2014; Balaban et al., 2021). In some cases, as in the model for *E. coli.* DSM 4415, for example, the antimicrobial effect of specific phenolic compounds was not clear, since the model's performance was affected by the presence of other molecules, such as anthocyanins, present in the PPE samples with no antimicrobial activity. This aspect might be related to the relatively low number of PPE samples tested; consequently, the result from PLS-DA should be considered as indicative. Further studies are thus required to confirm these results in which single or mixtures of PPE components are tested. Nonetheless, the recurrent correlation with the "High" or "Medium" PLS-DA classes of certain bioactive compounds, such as the punicalagin isomers, epicatechin, catechin and chlorogenic acids, strongly suggests them to be responsible for the antimicrobial activity of pomegranate peel extracts.

The combination of natural compounds with high contents of bioactive molecules in bio-based films is proven to enhance the activity of edible coatings and reinforce food product conservation. Among these, PPEs have been widely used alone or combined with edible films for food conservation as a source of bioactive compounds. A study showed that the combination of different PPE concentrations within bio-based films provides a biofunctional edible film for the packaging process. In similar studies, the addition of PPE to edible films or its usage as food preservatives resulted in high antioxidant activity and significant antibacterial ability against several bacterial strains (Yuan et al., 2015; Chen et al., 2020), including *L. monocytogenes* (Takó et al., 2020), *S. aureus, E.coli* (Emam-Djomeh et al., 2015; Ali et al., 2019), *B. cereus*, and *S. Typhimurium* (Hanani et al., 2018). Thus, based on the antimicrobial activity that was observed in the current study, some of the PPEs had high efficiency and potential applications in the food industry as a natural alternative to chemically synthetic antimicrobial agents against food-borne bacteria.

Previous studies have confirmed water extracts of pomegranate peel exhibit significant antimicrobial activity against both Gram-positive and Gram-negative bacteria (Chen et al., 2020; El-Beltagi et al., 2022). While the present study similarly revealed water extracts to exert strong antimicrobial activity against *S. aureus* and *L. monocytogenes* strains, very weak antimicrobial activity was observed against *Sa. bongori* DSM 13772 and *E. coli* strains. This goes along with the results of previous studies where aqueous PPE showed no antimicrobial activity against several gram-negative bacteria like *E. coli, P. mirabili* and *K. pneumonia* (Chebaibi and Filali, 2013). Sadeghian et al. (2011) reported that both aqueous and methanolic extracts showed good antibacterial activity against *S. aureus* and *P. aeruginosa*, however, the methanolic extract had higher antimicrobial activity against all tested bacteria. The same was observed in a different study where aqueous and methanolic extracts of PPE were proven to be efficient against *P. aeruginosa, S. marcescens, E. coli*, or *K. pneumoniae* bacteria (El-Beltagi et al., 2022). Another study demonstrated that the aqueous PPE had a significantly higher inhibitory effect on the growth of *Salmonella* when compared to juice powder extract (Wu et al., 2022). Other past studies reported comparatively weaker antimicrobial activities for water extracts compared with those of acetone and/or methanol. The inconsistencies in findings between studies might be explained by various differences between studies, including pomegranate cultivars, different methods of extraction and extraction solvent polarities, and the different type of bacterial species/strains (Devatkal et al., 2013; Nozohour et al., 2018; Balaban et al., 2021; Abdel Fattah et al., 2022).

Regarding the protechnological strains, both *La. casei* Shirota and *Li. reuteri* 17938 showed less sensitivity to high concentrations or were not inhibited at all by the specific PPEs used in this study. These results agree with those by Alexandre et al. (2019), who reported promising results in relation to LAB, which was not inhibited by any of the tested PPE extracts. Indeed, the resistance of LAB to these compounds could be considered advantageous since these bacteria are potentially beneficial for human health. This finding could be

of interest to the dairy industry; for example, the application of PPE could increase the antioxidant activity of dairy products, reducing the risk of contamination by foodborne pathogens, while leaving the stability of naturally present LAB undisturbed. Indeed, Al-Hindi and Abd El Ghani (2020) confirmed that the addition of pomegranate peel polyphenol extracts to fermented dairy products did not cause the LAB density to fall below the functional levels needed for exerting health benefits. Both LAB and phenolic compounds are advantageous for human health, so it might be convenient to develop novel function-enhanced food products that intentionally contain both components. A further study found that PPE-containing films used for packaging specialty cheeses helped maintain the LAB cultures during storage; in fact, they enhanced their populations increased (Mushtaq et al., 2018). However, in a study conducted by Abd El-Aziz et al. (2013), it was reported that low concentrations of the aqueous PPE stimulated the growth of starter LAB, contrastingly, it was also observed that the gradual increase of the PPE can inhibit the bacterial growth and vitality of starter LAB. Based on this, further in-depth studies would always be required to assess the suitability of these extracts for any practical applications of such technology.

Biofilm-associated bacteria are a huge issue where with ease they can resist against host defences, antimicrobial agents, and other stresses in comparison to planktonic cells (Fink et al., 2018). The present study also investigated the susceptibility of microbial biofilms to PPEs. The results showed that PPEs were able to inhibit biofilm development at concentrations below the MIC of the tested isolates. Benslimane et al. (2020) reported the inhibition of biofilm formation by PPE for all the bacteria strains tested in their study, and the level of inhibition increased by increasing extract concentrations. In the present study, significant reductions were also obtained at much lower concentrations of water-extracted PPE, with more than 70% reduction in biofilm formation observed at a concentration of 0.75 mg/mL for *S. aureus* strains and 0.75–3.00 mg/mL for *L. monocytogenes* strains.

A recent study investigated the effect of different extraction solvent types and methods on the antibacterial and antibiofilm activities of PPE and reported that water extract concentrations of 12.5 and 6.25 mg/mL reduced biofilm formation by 94% and 96%, respectively (Nasreddine et al., 2018). More other studies reported the PPE antibiofilm activity against several pathogens, including *B.cereus* strain where biofilm was removed at high ratios ranging between 79% and 83% (Balaban et al., 2021). The *C. albicans* and *Streptococcus parasanguinis* where biofilms were inhabited by 55% and 62%, respectively (De Almeida Rochelle, 2016). In addition that up to 35% of *E. coli* biofilm biomass could be removed by PPE (Fink et al., 2018).

The antibiofilm activity of PPEs could be attributed to the presence of phenolic compounds, such as punicalagin and ellagic acid, which might exert their effects through different mechanisms of action (Balaban et al., 2021). It was observed that methanolic pomegranate extract rich in ellagic acid was able to inhibit the formation of biofilm of *S. aureus*, MRSA, *E. coli*, and *C. albicans*. Phenolic compounds inhibit bacterial biofilm formation by suppressing different regulatory mechanisms: they can alter bacteria performance by reducing its motility, decreasing adhesion, blocking the expression of virulence factors associated with bacteria pathogenicity, and intervening with the mechanism of cell-substratum attachment by modulating the surface charge of bacteria (Dubreuil, 2020; Ebrahimnejad et al., 2020; Takó et al., 2020). Thus the results of this study indicated PPEs' antibacterial and antibiofilm potentials that might be used as food preservatives in food industries and contribute to food waste reduction.

In the context of Italy, the good antimicrobial activities of PPEs obtained in the present study from Sardinian varieties of the fruit support the carrying out of further research into the pomegranate germplasm from local sources and its valorization**.**

5. Conclusions

The current study contributes to furthering our knowledge of the phenolic composition of pomegranate peel extracts from two internationally-known varieties, Wonderful and Mollar de Elche, a commercially relevant Italian variety, Primosole, and four lesser-known Sardinian varieties, Sassari 1, Sassari 2, Sassari 3, and Arbara Druci. Expanding the study to include local varieties was important from the perspective of Italian plant breeding and the valorization of local biodiversity. Detailed characterization of the bioactive components of peel extracts from specific varieties of pomegranate is necessary in order to explain their antimicrobial activity against some of the most common pathogens such as *S. aureus*, *L. monocyogenes*, and *E. coli*. Three pomegranate varieties were shown to exhibit strong antimicrobial activity: Mollar de Elche, Primosole, and Sassari 3. The first two varieties were shown to be rich in punicalagins, flavonoids, and chlorogenic acid, the presence of which could account for their antimicrobial activities. On the other hand, the overall low to medium level of phenolic compounds in Sassari 3, with the exception of chlorogenic acid, lead us to hypothesize that this variety's anti-microbial properties are more likely to arise from the combination and synergic action of specific molecules. In conclusion, the results from this study support the valorization of pomegranate peel, an agro-industrial waste product, in the view of ecological sustainability and circular economies.

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Conflict of Interest

The authors have no competing interests to declare.

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