Antimicrobial and antibiofilm activities of pomegranate phenolic compounds against foodborne pathogenic bacteria

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This Ph.D. thesis research project is aiming to investigate the antimicrobial, antibiofilm, and anti-quorum sensing capacities of the pomegranate peel extracts (PPE) on several foodborne pathogenic bacteria, together with analyzing the chemical composition of the PPE’s bioactive compounds and their antioxidant activities. Bioassay-guided fractionation was used for identifying the most active natural products in the top-performing extracts. These fractions were then assessed for cytotoxicity using LDH cytotoxicity assay against human keratinocytes (HaCaTs) was performed. Finally, the effectiveness of the top-performing extracts on food matrix against *Staphylococcus aureus* was evaluated.

**Le attività antimicrobiche e antibiofilm dei composti fenolici della buccia di melograno contro i batteri patogeni di origine alimentare**

Questo progetto di ricerca di tesi di dottorato ha lo scopo di indagare le capacità di rilevamento antimicrobico, antibiofilm e anti-quorum degli estratti di buccia di melograno (PPE) su diversi batteri patogeni di origine alimentare, insieme all'analisi della composizione chimica dei composti bioattivi del PPE e delle loro attività antiossidanti. Il frazionamento guidato dal saggio biologico è stato utilizzato per identificare i prodotti naturali più attivi negli estratti con le migliori prestazioni. Queste frazioni sono state quindi valutate per la citotossicità utilizzando il test di citotossicità LDH contro i cheratinociti umani (HaC, aTs). Infine, è stata valutata l'efficacia degli estratti più performanti sulla matrice alimentare contro lo *Staphylococcus aureus.*

**Key words**: Punica granatum, agrobiodiversity, OPLS-DA, punicalagin, *Staphylococcus aureus*

# **1. Introduction**

In accordance with the Ph.D. thesis project previously described (Amira, 2022), this oral communication reports the main results of the conducted activities concerning:

**A1.** Extraction of PPEs from pomegranate fruit peels.

**A2.** Determination of the antimicrobial activity of the PPE.

**A3.** Determination of antibiofilm and anti-quorum sensing.

**A4.** Phytochemical composition analysis of the PPE bioactive compounds.

**A5.** Bioassay-guided fractionation approach for isolating the most active natural products in the top-performing extracts.

**A6.** Cytotoxicity assessment of human cells using LDH cytotoxicity assay of treated human keratinocytes (HaCaTs).

**A7.** Testing the effectiveness of the top-performing extracts on food matrix against staphylococcus aureus.

Pomegranate (*Punica granatum* L.) is one of the oldest species of domesticated fruit world (Schwartz et al., 2009) which is rich in bioactive phytochemicals known for their antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Its peel 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 (Puneeth and Chandra, 2020; Gigliobianco et al., 2022). Hydrolyzable ellagitannins are the predominant phenolic compounds in pomegranate peel, and they are also the constituent phytochemicals exhibiting the highest antioxidant capacities (Gigliobianco et al., 2022). Moreover, the magnitude of the antioxidant and antitumor activities of pomegranate peel extract are 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 (Celiksoy and Heard, 2021). Notably, food poisoning is predominantly linked to bacterial contamination by Gram-negative bacteria and Gram-positive bacteria (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. (Slobodníková et al., 2016). Thus 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 (Xu et al., 2017).

# **2. Materials and Methods**

The chemical composition and antimicrobial activities of the peel from seven pomegranate varieties were evaluated. Dried bulk specimens were ground into a fine powder and extracted by aqueous decoction in two different levels of temperature and maceration in ethanol of the seven different pomegranate varieties. PPEtotal phenolic content was assessed by Folin-Ciocalteu assay where total flavonoids were quantified by colorimetric assay according to the AlCl3 method and analysis of condensed tannins was carried out by vanillin assay. The antioxidant capacity of pomegranate extract was evaluated using both DPPH and ABTS methodologies and finally, Reverse-phase HPLC analysis of phenolic compounds was performed using an Agilent 1100 Liquid Chromatography (LC) system.

The antimicrobial activities of PPE were quantitatively evaluated in vitro by measuring the Minimum inhibitory concentrations (MICs) of the seven PPEs were estimated on different foodborne pathogenic bacteria. The antibiofilm activity was assessed using a crystal violet (CV) assay with some modifications. Bioassay-guided fractionation approach for isolating and identifying the most active natural products in the top-performing extracts, using PREP-HPLC with the goal of the identification of a single bioactive compound and Putative matches of compounds elucidation via LC–FTMS for the best-performing extracts. Cytotoxicity assessment of human cells using LDH cytotoxicity assay of treated human keratinocytes (HaCaTs) was performed. Finally, the effectiveness of the top-performing extracts on food matrix against *staphylococcus aureus* was evaluated.

# **3. Results and Discussion**

## **3.1 chemical composition of PPE**

The data show wide content variability between cultivars, In agreement with Whang et al. (2011), we found extraction with water at 40 °C for 4 hours to be an efficient method for the extraction of pomegranate peel antioxidants However, our data also show that extraction at this higher temperature was not always accompanied by a higher concentration of extracted compounds compared with extraction at room temperature tab 1. Eighteen phenolic compounds were identified by HPLC-DAD, and the levels of total phenols, flavonoids, and tannins are in line with the previous literature. Saad et al. (2012). Two out of the seven tested cultivars were identified as the varieties that differed from the others the most. To highlight the varietal-specific features of PPE further, models were generated investigating a single variety vs all others. Specifically, three PPEs stood out for their overall low concentration of phenols, tannins, and flavonoids. On the other hand, two PPEs were distinguished by their absence of anthocyanins and good presence of flavonoids and phenolic acids, specifically catechin, rutin, and caffeic acid, and two varieties presented high concentrations of punicalagin isomers.

The antioxidant activities of PPEs obtained at the two different extraction temperatures were generally similar. The differences in values obtained between the two extraction temperatures were even lower for DPPH values,. As for ABTS, two varieties were demonstrating the greatest differences, with hot water extraction resulting in higher phenol concentrations (Fig. 1). Previous studies have attributed the main antioxidant activity of PPE to punicalagins, punicalins, and ellagic acids (Rosas-Burgos et al., 2017). In contrast, our findings attributed a negligible role to the latter two compounds and only a secondary role to punicalagins. Instead, our results indicate 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.

**3.2 antimicrobial activity of PPE**

Most of the PPEs showed some effectiveness at suppressing microbial growth, in addition, the MIC values recorded in the present study (3 to 0.09 mg mL-1) were much lower than those reported in previous studies (Wafa et al., 2017; Nasreddine et al., 2018).and Gram-negative bacteria notably more resistant than Gram-positive bacteria this is in agreement with results obtained from (Alexandre et al., 2019). PLS-DA analysis enabled us to identify the bioactive compounds contributing the most to the antimicrobial activities of PPE. Table 1 reports the molecules associated with the antimicrobial activity for each microbial strain.

**Table 1** *List of the variables mostly related to the antimicrobial activity of the PPE according to the PLS-DA models performed per each bacterial strain tested*.

|  |  |
| --- | --- |
| **Strain** | **Variables** |
| S. aureus 20231 | Chlorogenic acid, Total Tannins, Punicalagin β, Punicalagin α |
| S. aureus 2569 | Total Tannins, Total Phenols, Epicatechin, Total Flavonoids |
| S. aureus 6948 | Punicalagin β, Punicalagin α, Catechin, Total Flavonoids, Chlorogenic acid |
| L. monocytogenes 15675 | Chlorogenic acid, Total Phenols, Epicatechin, Punicalagin α, Total Tannins, Punicalagin β |
| L. monocytogenes 20600 | Total Tannins, Total Flavonoids, Chlorogenic acid, Punicalagin β, Epicatechin, Punicalagin α  |
| E. coli 4415 | Punicalagin α, Punicalagin β, Total Tannins, Rutin, Catechin |
| Lac. Paracasei Shirota | Total Tannins, Epicatechin, Total Flavonoids, Total Phenols, Punicalagin α, Punicalagin β, Rutin |
| Lim. Reuteri 17938 | Epicatechin, Total Flavonoids, DPPH, Caffeic acid, Punicalagin α, Punicalagin β,  |



**Figure 1** *OPLS loading scatter plots representing the relationships between the X variables (PPE chemical composition) and the Y variable: DPPH (a) and ABTS (b). The variables highlighted in bold blue type are those strongly correlated with the Y variable (in terms of variable importance on projection: VIP).*

**3.2 antimicrobial activity of PPE**

The results showed that PPEs were able to inhibit biofilm development at concentrations below the MIC of the tested isolates (Fig. 2). 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 65% reduction in biofilm formation observed at a concentration of 3 to 0.75 mg/ml for Gram-positive bacteria strains. The antibiofilm activity of PPEs could be attributed to the presence of phenolic compounds such as punicalagin and ellagic acid, which may exert their effects through different mechanisms of action (Balaban et al., 2021a).



**Figure 2**  *The effect of the three cold water PPE (from ME, PS, SS3 cultivars), applied at different concentrations (MIC1), on biofilm formation. Letters above the columns indicate statistical differences according to Tukey’s test.*

# **4. Conclusions and Future Perspectives**

The current study contributes to furthering our knowledge of the phenolic composition of pomegranate peel extracts in different national and international varieties, 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, antibiofilm, and antioxidant activities against some of the most common pathogens. Three pomegranate varieties were shown to exhibit strong antimicrobial activity. Those varieties were shown to be rich in punicalagins, flavonoids, and chlorogenic acid, the presence of which can account for their antimicrobial activities. In conclusion, this study proposes a formulation of pomegranate peel extract that valorizes an agro-industrial waste in the context of sustainability and circular economy. Pomegranate extracts should be considered as potential sources of natural, plant-derived antimicrobials, providing an alternative to artificial antimicrobial products.

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