**Chapter N (please do not write anything in this line. Editors will annotate the chapter number)**

**Valorisation of pomegranate waste and by-products for new models of circular economy**

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**Abstract.** In the current context of affirmation of the concept of circular economy as a new paradigm of sustainability, the present research aimed at the enhancement and innovation for the supply-chain of pomegranate. In the perspective of optimizing a multifunctional platform, two samples were studied obtained from pomegranate waste: a lyophilized aqueous extract from pericarp and a micronized powder obtained by grinding the pericarp after freeze-drying. The HPLC-DAD-MS chemical characterization revealed a content of hydrolyzable tannins of 23.1% by weight for the lyophilized extract and 20.8% for the micronized pericarp. The antimicrobial activity was evaluated on five fungi of agricultural and food interest (*Alternaria* sp., *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Mucor* sp., *Penicillium digitatum* and *Pythium ultimum*). In all cases, we observed an inhibition of the fungal mycelium proportional to the sample concentrations, with average higher efficacy for the lyophilized extract compared to the micronized powder. These results will allow to assess the possible applications of waste and by-products from pomegranate processing to obtain, within integrated platforms for the recovery of active ingredients with functional properties, innovative semi-finished and finished products for uses in diversified commodity sectors.

**Keywords.** Pomegranate, Circular economy, Multifunctional platform, Hydrolysable tannins, Natural antimicrobials, Natural antioxidants.

# 1 Introduction

Pomegranate (*Punica granatum* L.) is a dicotyledonous angiosperm plant belonging to the *Punicaceae* family, which is widespread mainly in arid and semi-arid regions of Iran, the Himalayas and the north of India, China, United States and the entire Mediterranean region thanks to its ability to adapt to adverse conditions. The varieties widespread in Italy, such as Tondo verde, Dolce di Sicilia, Dente di Cavallo, are more suitable for the climate of the specific area (Passafiume et al., 2019). In recent years, scientific studies have shown a preventive action of the fruit and its extracts against numerous chronic and genetic diseases such as cancer, type 2 diabetes, arteriosclerosis and cardiovascular diseases (Bar-Ya’akov, I. et al., 2019; Caruso et al., 2020). Positive effects were highlighted not only concerning the edible part of the fruit, but also the inedible fraction, which currently represents a waste from the processing of fruits and has a higher content of bioactive compounds (Mastrogiovanni et al., 2020). The attention of numerous studies is focusing precisely on these wastes, which represent 48% by weight of the whole fruit and are an important source of bioactive substances such as polysaccharides, flavonoids, phenolic acids and other polyphenols (Romani et al., 2012; Joshi et al., 2019; Passafiume et al., 2019). The biological properties of polyphenols in pomegranate waste materials together with a greater awareness of sustainability, and the need to implement a circular economy approach, are leading to focus attention on this plant species for the use of its waste and by-products in different sectors. The implementation of multifunctional platforms following a biorefinery approach, integrating different sustainable and green processes aimed at exploiting the whole biomass to obtain different new bio-based products such as chemicals, materials, semi-finished products up to bio-fuels and energy, is nowadays the most complete application of the circular economy concepts in an industrial perspective (Lucarini et al., 2018). In this context, the present study is aimed at optimizing a multifunctional platform for the enhancement and innovation of the supply-chain of pomegranate. In order to assess the pre-feasibility, two samples were studied obtained from pomegranate waste: a lyophilized aqueous extract from pericarp (Lyophilized Extract, LE) and the micronized powder obtained by grinding the pericarp after freeze-drying (Micronized Pericarp, MP). The raw material used to prepare the samples were the waste fruits because of insufficient size or with characteristics that make them unsuitable for sale as a fresh product or for obtaining juice, furnished by Supreme Fruit srl (Cisterna di Latina LT, Italy). LE and MP were chemically characterized for their content in bio-active hydrolyzable tannins by HPLC-DAD-MS analysis; then their antimicrobial activity was tested on five fungi of agricultural and food interest (*Alternaria* sp., *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Mucor* sp., *Penicillium digitatum* and *Pythium ultimum*). These results will allow to assess possible applications of waste and by-products from pomegranate processing to obtain, within integrated platforms, innovative semi-finished and finished products by the recovery of active ingredients with functional properties to be used in diversified commodity sectors.

# 2 Materials and methods

**2.1 Chemicals**

All solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Sigma Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, USA). Gallic and ellagic acids, of analytical grade, were purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France). HPLC-grade water was obtained via double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, USA). The PDA (Potato Dextrose Agar) was purchased from VWR International (Radnor, Pennsylvania, USA).

**2.2 Samples**

The samples under study were obtained from waste fruits furnished by Supreme Fruit srl (Cisterna di Latina LT, Italy). For micronization, the pomegranate pericarp was cut into small pieces, frozen, freeze-dried, then finely grinded with a laboratory horizontal blade mill with a rotation speed of 20,000 rpm (IKA), with short pulses to avoid temperature increases in the material due to friction and impact with the blades. For the extraction, the plant material was placed in a teflon filter to avoid the release of non-soluble polysaccharides in the extract, and kept under stirring in deionized water (6.5% w/v) at 90°C for 1 hour; the material was then left to macerate at room temperature for 12h. The extract was rinsed to an exact final volume, then centrifuged at 5000 rpm for 10 min and analyzed. The aqueous extract was then weighed, frozen in Petri dishes and lyophilized.

**2.3 HPLC-DAD-ESI-MS analysis**

A HP-1260 liquid chromatograph was used, equipped with a DAD detector and a MSD API-electrospray (Agilent Technologies, Santa Clara, CA) set in negative ionization mode. A Luna, C18 250×4.60 mm, 5 μm column (Phenomenex, Torrance, CA), operating at 26 °C was used. Eluents: H2O (pH 3.2 by HCOOH) and CH3CN. A four-step linear solvent gradient from 100% H2O up to 100% CH3CN was performed as previously described (Romani et al., 2012). Mass spectrometer operating conditions: gas temperature 350 °C, flow rate 10.0 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C and capillary voltage 3500 V. Fragmentor 120 eV. Tannins were identified by comparing their retention times, UV-Vis and mass spectra with those of the commercial standards. Compounds were quantified in HPLC/DAD by using five-point regression curves (r2 ≥0.9998) in gallic and ellagic acids. Analyses were carried out in triplicate, the results are reported as mean values with standard deviations ≤ 5%.

**2.4 *In-vitro* test**

The test was conducted against five fungi of agricultural and food interest: *Alternaria* sp., *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Mucor* sp., *Penicillium digitatum* e *Pythium ultimum*) using the poisoned food technique, where the substrate (Potato Dextrose Agar, PDA) is incorporated with the extract to test and the colony diameter is registered, obtaining at the end an inhibitory value expressed as percentage. For the experiment purpose, the PDA was prepared following the label instruction and additionated with the extracts in the amount of the 0% (control), 0.5%, 1.0% and 2.0% w/v, then the pH was corrected to 5.6 using KOH 1M and then autoclaved at 121°C for 21 minutes. After the sterilization, the molten substrate was dispensed in 55mm Petri dishes. The inoculation was done placing in the center of each Petri dish a 6mm mycelium plug obtained from colonies in active growth. The test is terminated as soon as the mycelium of at least a colony arrives at the end of the Petri dish. The diameter of each colony is measured and the inhibitory percentage was calculated using the formula (Cd - Td)/Cd\*100, where Cd = control diameter and Td = treated diameter.

**3 Results and discussion**

The samples under study were obtained from waste of pomegranate processing, in particular from fruits of insufficient size or with characteristics that make them unsuitable for sale as a fresh product or for obtaining juice. The pericarp was isolated and used as a raw material to obtain the MP and the LE as described in the “Materials and methods” section.

**3.1 HPLC-DAD-MS characterization of the tannin content**

For the quali-quantitative chemical characterization of the tannin content, the HPLC-DAD-MS analysis was performed on both the two samples under study and the aqueous extract of pericarp before lyophilization. The results obtained for the aqueous extract of pericarp are reported in Table 1. The extraction process appears to preserve unaltered a significant portion of α- and β-punicalagin, hydrolysable tannins representative of the *Punica granatum* species. The summed amounts of the respective hydrolysis products, α- and β-punicalin, represents 18% by weight with respect to the quantity of the two non-hydrolyzed tannins.

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| Table 1. HPLC-DAD-MS analysis of the hydrolyzable tannins in the aqueous extract of pomegranate pericarp before lyophilization. | | |
|  | mg/mL of extract | mg/g plant material |
| HHDP glucose | 0.012 | 0.185 |
| Monogalloyl glucose | 0.009 | 0.14 |
| Gallic acid | 0.035 | 0.565 |
| α-punicalin | 0.067 | 1.073 |
| β-punicalin | 0.087 | 1.386 |
| α/β-punicalagin isomer I | 0.224 | 3.582 |
| α/β-punicalagin isomer II | 0.082 | 1.307 |
| α-punicalagin | 0.274 | 4.379 |
| β-punicalagin | 0.578 | 9.248 |
| Galloyl-HHDP glucose | 0.008 | 0.131 |
| Ellagic acid hexoside | 0.008 | 0.133 |
| Vanoleic acid bilactone | 0.008 | 0.13 |
| Granatin B | 0.066 | 1.05 |
| Ellagic acid rhamnoside | 0.001 | 0.016 |
| Ellagic acid penthoside | 0.002 | 0.025 |
| Ellagic acid | 0.045 | 0.722 |
| **Tot** | **1.505** | **24.072** |

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| Table 2. HPLC-DAD-MS analysis of the hydrolyzable tannins in the Lyophilized Extract (LE) and Micronized Pericarp (MP). Data expressed in mg of compounds per gram of sample. | | |
|  | Lyophilized Extract (LE) | Micronized Pericarp (MP) |
| HHDP glucose | 3.936 | 0.276 |
| Monogalloyl glucose | 0.37 | 0.756 |
| Gallic acid | 5.891 | 0.638 |
| α-punicalin | 13.633 | 7.269 |
| β-punicalin | 14.243 | 9.848 |
| α/β-punicalagin isomer I | 38.262 | 0.975 |
| α/β-punicalagin isomer II | 9.874 | 1.257 |
| bis HHDP-hexoside isomers | 0.000 | 12.909 |
| α-punicalagin | 46.771 | 55.115 |
| β-punicalagin | 79.034 | 72.449 |
| Galloyl-HHDP glucose | 2.141 | 0.528 |
| Ellagic acid hexoside | 1.177 | 1.511 |
| Vanoleic acid bilactone | 1.093 | 0.000 |
| Granatin B | 8.102 | 39.999 |
| Ellagic acid rhamnoside | 0.334 | 0.224 |
| Ellagic acid penthoside | 0.501 | 0.379 |
| Ellagic acid | 6.192 | 4.063 |
| **Tot** | **231.554** | **208.196** |

The quali-quantitative characterization of the tannin content in LE and MP is reported in Table 2. The total tannin contents are similar for LE and MP, but it must be also considered that MP is the grinded vegetal tissue (still containing the fraction of fiber and other insoluble compounds) to be used as such, while LE is totally water soluble. The relative amount of punicalin with respect of α- and β-punicalagin is almost preserved during lyophilization (18% aqueous extract *vs* 22% LE), whereas MP, that didn’t undergo hot water extraction, showed a slightly better preservation of punicalagin content (13% total punicalin *vs* total punicalagin) that is anyway sufficient in both cases.

**3.1 *In vitro* test**

In all cases an inhibitory effect proportional to the sample dose was observed (Table 3), with averagely a better efficacy for LE than MP.

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| Table 3. Inhibitory value (%) for each pathogen and extract concentration. | | | | | | |
|  | Micronized Pericarp (MP) | | | Lyophilized Extract (LE) | | |
|  | 0.5% | 1.0% | 2.0% | 0.5% | 1.0% | 2.0% |
| *Alternaria* sp. | 17.02 | 36.17 | 44.68 | 13.48 | 31.91 | 59.57 |
| *Fusarium oxysporum* f. sp. *radicis-lycopersici* | 30.34 | 47.57 | 55.06 | 46.07 | 59.55 | 66.29 |
| *Mucor* sp. | 58.00 | 62.67 | 68.00 | 54.00 | 68.00 | 74.00 |
| *Penicillium digitatum* | 58.96 | 83.58 | 97.76 | 78.36 | 93.28 | 97.76 |
| *Pythium ultimum* | 20.00 | 63.33 | 70.00 | 36.00 | 88.00 | 100.00 |

For what concerns the fungi of food interest, the observed inhibition percentage obtained with MP ranged from 58.0 to 68.0% against *Mucor* sp. and from 58.9 to 97.7% against *Penicillium digitatum*, while the inhibition caused by LE varied from 54.0 to 74.0% against *Mucor* sp. and from 78.3 and 97.7% against *Penicillium digitatum*. The two samples were on average slightly less effective against fungi of agricultural interest. MP caused an inhibition from 17.0 up to 44.6% against *Alternaria* sp., from 30.3 to 55.0% against *Fusarium oxysporum* f. sp. *radicis-lycopersici* and from 20.0 to 70.0% against *Pyhtium ultimum*. The inhibition observed with LE ranged from 13.4 to 59.0% against *Alternaria* sp., from 46.0 to 66.2% with *Fusarium oxysporum* f. sp. *radicis-lycopersici* and from 36.0 to 100% with *Pythium ultimum*.

**4 Conclusions and future perspectives**

These results highlight the possibility of exploiting the waste from processing of pomegranate in an efficient way, to obtain new semi-finished and finished products rich in bioactive natural compounds with antimicrobial activity for food and agronomics, in a circular economy and sustainability perspective. The two up-scaled processes could be integrated in industrial platforms and biorefineries aimed not only at obtaining the primary products, but also at transforming the pomegranate waste material into a new and renewable resource with high added value, useful for applications in diversified commodity sectors.

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