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Aglianico grape pomace as a source of antioxidant and anti-proliferative biomolecules: Eco-friendly extraction and HRMS/MS-based molecular networking

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ABSTRACT

Grape pomace (GP), a by-product of the wine supply chain process, contains bioactive molecules with known healthy properties. This study examines the impact of different extraction techniques on three GPs of Aglianico *cultivar* [Cantine del Notaio, Barile, and Torrecuso]. Five eco-friendly extractive techniques [maceration (MAC), digestion (DIG), accelerated solvent extraction (ASE), microwaves (MW), and ultrasound (US)] were used with 50 % ethanol/water as solvent. Spectrophotometric assays showed that DIG and ASE extracts had the highest antioxidant activity and specialized metabolite content. Using the HRMS/MS-based molecular networking, DIG and ASE extract metabolome profiles were analyzed, identifying unknown compounds and known ones with validated antioxidant and chemopreventive effects. *In vitro* cell-based assay on HepG2 cells demonstrated that Barile GP DIG extract has the highest anti-proliferative activity. Hence, this work provides insight into the potential application of Barile GP DIG extract as a rich source of specialized metabolites with antioxidant and anti-proliferative activity.

1. Introduction

Most food-chain industrial processes work through a linear sequence of operations, often referred to as "take, make, dispose." However, this way of working leads to the generation of a huge amount of waste that typically ends up in landfills or incinerators (Lavelli, 2021). To design more sustainable production systems and thus improve resource management, numerous studies have therefore been initiated to give new life to food supply chain by-products, thus embracing the zero waste concept. The latter, indeed, sees waste as a valuable resource for learning how to manage products and recover all resources by applying a circular economy perspective (Gaur, Gurjar, & Chaudhary, 2022). A prominent agro-industrial sector for which it is important to apply this concept is the wine chain since it impacts global economies significantly and represents a high source of waste material and by-products to be valorized (Crescente et al., 2023).

The cultivation of the grape has reached a worldwide production of about 60 million tons per year (Bordiga, Travaglia, Locatelli, Arlorio, & Coïsson, 2015; Castellanos-Gallo et al., 2022). Europe has the largest vineyard surface in the world (33,000 Km^2), with Spain (9670 Km^2), France (7870 Km^2), and Italy (6950 Km^2) being the most concentrated (Lazzari & Piccarreta, 2023). Furthermore, Italy holds the third position in the global rankings for vineyard area, constituting 9 % of the total, trailing behind Spain and France. The Italian wine production system is complex and characterized by diverse supply and organizational forms of production. Notably, there are 408 wines with a PDO designation and

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118 with a PGI indication, a significantly higher number than Spain and France (Pomarici, Corsi, Mazzarino, & Sardone, 2021). Even if the winemaking process is generally regarded as environmentally friendly, it produces various residues, primarily grape pomace (GP), wine stems, and wine lees. Specifically, following alcoholic fermentation in the wine production process, approximately 10 %–30 % of the processed grapes' weight persists as solid organic waste, known as pomace, composed of stalks, skin, and seeds. Beyond the conventional practice of using GP as animal feed, various alternative uses for these valuable by-products have been explored (Abouelenein et al., 2023). One prominent alternative involves the extraction of bioactive molecules since it is known that approximately 70 % of the grapes' specialized molecules, mainly polyphenols (phenolic acids, tannins, stilbenes, flavan-3-ols, flavonols, anthocyanins, and chalcones), are still in the pomace after the vinification process. Given these molecules' anti-inflammatory, antioxidant, antimicrobial, cardioprotective, anti-cancer, and anti-aging properties, products derived from GP can be attractive for improving human health (Abouelenein et al., 2023; Cisneros-Yupanqui, Rizzi, Mihaylova, Deseva, & Lante, 2023; Coluccia, Valente, Fusco, De Leo, & Porrini, 2020).

Aglianico is considered among the most important wines in southern Italy, particularly the "Aglianico del Vulture," a traditional red wine characteristic of the Basilicata Region Vulture area with a PDO designation in agreement with the EU 1971 legislation. The Aglianico vineyards are located on the Vulture volcanic hillsides, whose peculiar tuffaceous composition and favorable climatic conditions give the wine a unique taste and smell (Cafaro et al., 2016). This grape denotes a peculiar ecosystem that is still being poorly investigated due to its specific winemaking condition; hence, the thought of investigating the biological and phytochemical composition of the GP starting from the comparison of different eco-friendly extractive methods, including conventional [such as maceration (MAC) and digestion (DIG)] and unconventional [such as microwave-assisted extraction (MW), ultrasound-assisted extraction (US), and accelerated solvent extraction (ASE)] extraction techniques (Bitwell, Indra, Luke, & Kakoma, 2023). MAC is a low-cost extractive method performed at room temperature and is thus recommended for heat-labile molecule extraction, while DIG represents a modified version of maceration, allowing a possible increase in extraction yield through low heating application. Either MAC or DIG enables the dissolution of bioactive compounds *via* diffusion from plant material to solvent; however, in the case of DIG, the heat applied during the extraction process allows the solvent's viscosity to be lowered, thereby facilitating the extraction of secondary metabolites (Abubakar & Haque, 2020). MW is considered a novel extraction method applicable to extracting fluid-soluble products from a wide variety of plant materials by exploiting microwave energy to heat the solvent and plant material directly, determining a localized heating and rapid disruption of plant cells. The principle of microwave heating is hinged on the direct interactions of microwaves with molecules through two main mechanisms: ionic conduction and dipole rotation. Ionic conduction involves the movement of ions caused by an applied electromagnetic field, resulting in the solution's resistance to this ion flow generating friction, which in turn produces heat (Eskilsson & Björklund, 2000). ASE is another novel extraction technique based on applying high pressure and high temperature to keep a solvent in a liquid state at temperatures above its normal boiling point. This elevated pressure enhances the extraction process, contributing to reduced extraction times and lesser solvent usage. Finally, another method used in the present investigation is UAE, which is based on the application of ultrasound waves which pass across the medium, generating alternating cycles of high and low pressure, resulting in the acoustic cavitation phenomenon. When cavitation bubbles collapse upon the plant material the inter-particle collision generate erosion, sonoporation, and cell rupture facilitating inorganic and organic molecules extraction from plant material (Azmir et al., 2013; Jha & Sit, 2022). All the cited techniques can be considered "green methods" applicable in recovering active compounds from by-products as they meet the Environmental Protection Agency (EPA) standard set, including using safe solvents and design for energy efficacy (https://www.epa.gov/greenchemistry/pubs/about_gc.html). Extracting specialized metabolites from food-chain by-products is an important current scientific field, increasingly oriented towards reducing environmental pollution. It is indeed recognized that food-chain waste, when improperly discarded, can released into the soil phenolic compounds, leading to ecological repercussions on plant development and growth, particularly during the germination stage (Misra, Dutta, Jha, & Ray, 2023).

When dealing with the extraction of secondary metabolites, besides the extraction technique, the choice of the solvent to be used is also important to minimize environmental emissions and preserve human health when producing extracts for nutraceutical, food, or cosmetic fields. Among the approved solvents for formulating functional foods by the European Food Safety Authority (EFSA) are ethanol, ethyl acetate, and acetone (Gullon et al., 2018). While these organic solvents have conventionally been considered safe and advantageous, there is a rising interest in identifying more eco-friendly alternatives. This interest stems from the need to decrease the emission of volatile organic compounds associated with these solvents, which have been recognized contributors to global warming (Gómez-Cruz, Cara, Romero, Castro, & Gullón, 2020). In the case of GP extraction, most of the studies used acidified organic solvents like methanol (De la Cerda-Carrasco, López-Solís, Nuñez-Kalasic, Peña-Neira, & Obreque-Slier, 2015; Fuchs et al., 2020; Negro, Aprile, Luvisi, De Bellis, & Miceli, 2021). It was demonstrated that, albeit toxic, methanol is mostly effective in extracting polyphenols with low molecular weight, while aqueous/acetone mixtures are more linked with extracting flavonols with high molecular weight (Goula, Thymiatis, & Kaderides, 2016). On the other hand, the acidic environment can enhance plant material degradation and improve polyphenols extraction rate from the matrix when the acid is present in low concentrations, as high mineral acid concentrations (1–2 M) may degrade labile phenolic molecules (Novak, Janeiro, Seruga, & Oliveira-Brett, 2008). In the case of the present investigation, it was decided to use environmentally friendly solvents like 50 % ethanol/water.

Extracts were characterized by HRMS/MS-based molecular networking to identify promising antioxidant and anti-proliferative specialized metabolites within the metabolic profile of Aglianico GP. The application of an MS-based molecular network (Teta et al., 2022) allowed a detailed characterization of the Aglianico GP metabolome, demonstrating the effectiveness of this approach in swiftly dereplicating complex natural mixtures without requiring complicated and time-consuming chromatographic purifications.

As far as is known, only one investigation has evaluated Aglianico GP extraction, biological activity, and phytochemistry (Caponio et al., 2022). For this reason, the present study aims to compare different extractive techniques' effects on three Aglianico GPs from "Aglianico del Vulture" (Cantine del Notaio), Aglianico of Barile, and Aglianico from Torrecuso. The efficiency of the chosen extractive methods in recovering specialized metabolites was evaluated using three different complementary antioxidant assays (TPC, FRAP, and DPPH tests). This allows for identifying the most promising extraction technique between the chosen conventional and unconventional ones. These latter were analyzed for the content of specialized metabolites, employing an innovative HRMS/MS-based approach as molecular networking, as well as anti-proliferative activity by *in vitro* cell-based assay.

Hence, this work addresses the dual goals of waste valorization in the wine industry and the development of high-value applications for nutraceutical and pharmaceutical industries.

2. Material and methods

2.1. Plant material

Samples were collected between October and November 2019 from three different wineries based on seasonal availability. At "Cantine del

Notaio" in Rionero in Vulture (Potenza, Italy), the pomace is derived from locally grown Aglianico grapes that are finely ground and undergo fermentation at room temperature (22–26 °C) for 6 days. In Barile (Potenza, Italy), pomace is obtained from Aglianico grapes after domestic wine production, where they are finely ground and fermented at room temperature for 16 days in plastic tanks. In Torrecuso (Benevento, Italy), pomace originates from Aglianico grapes that are finely ground and fermented at room temperature for 7 days. The Aglianico grape pomace was harvested after the final racking stage, pressed after fermentation, dried for 6 h in an oven at 55 °C, crushed with a mortar and pestle, vacuum-sealed, and stored at −20 °C.

The initial humidity percentage of "Aglianico del Vulture" (Cantine del Notaio), Aglianico of Barile, and Aglianico from Torrecuso GP was 35.72 % ± 9.13. After drying for 6 h in an oven, disposed in a thin layer at 55 °C, the final humidity percentage was 2.05 % ± 0.91. Hence, the three GP varieties were subjected to extraction procedures.

2.2. Extraction techniques

The optimization of optimal extraction conditions was evaluated by making a comparison between four different extractive methods. Each extraction was performed in triplicate, with fresh solvent added three times until complete exhaustion of the drug. The three extracts were combined, filtered using a vacuum pump with 40 nm filters, dried using a rotary evaporator, frozen, and lyophilized. The extraction yield (%) was calculated as the weight of dried extract divided by the weight of the initial material, multiplied by 100.

2.2.1. Maceration (MAC)

Dried grape pomace (15 g) was macerated in the dark and under agitation in a hydroalcoholic mixture (50 % ethanol/water 150 mL) for 2 h at 37 °C temperature.

2.2.2. Digestion (DIG)

Dried grape pomace 15 g was extracted at 60 °C under agitation for 2 h using a hydroalcoholic mixture (50 % ethanol/water, 150 mL). The extraction was performed in the dark.

2.2.3. Ultrasound-assisted extraction (US)

15 g of dried grape pomace was sonicated for 2 h in a hydroalcoholic mixture (50 % ethanol/water 150 mL) in a glass beaker, using a Bransonic M1800-E sonicator (potency output = 70 W; frequency = 40 KHz). Temperature readings were every 30 min and ranged from 25 °C to 40 °C.

2.2.4. Microwave-assisted extraction (MW)

15 g of dried grape pomace were mixed with 150 mL of 50 % ethanol/water solvent in a glass beaker covered with parafilm. The mixture was subjected to extraction in a microwave oven (200 W) for 25 min, followed by cooling at room temperature for an additional 5 min. The process was repeated, resulting in a total extraction time of 1 h.

2.2.5. Accelerated solvent extraction (ASE)

5 g of dried wine pomace was extracted with an accelerated solvent extractor (ASE 150, Dionex), using a hydroalcoholic mixture (50 % ethanol/water) as solvents at $P = 1500–1600$ psi and $T = 120$ °C (3 static extraction cycles of 5 min).

2.3. Antioxidant activity evaluation

The total phenol content (TPC) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay and Ferric Reducing Antioxidant Power assay (FRAP) assay were performed using the protocols of Faraone et al. (2022) (Faraone et al., 2022). For TPC, data were reported as mg of Gallic Acid (GAE) equivalents per g of dried extract (mgGAE/g), considering the calibration curve of GAE. For DPPH and FRAP, data were reported as mg of Trolox (TE) equivalents per g of dried extract (mgTE/g), considering the calibration curve of TE.

Relative antioxidant capacity index (RACI) was evaluated following the method of De Rosa et al. (2024) (De Rosa, Ponticelli, Teta, Carlucci, Milella, Esposito, et al., 2024).

2.4. Total tannin content (TTC)

Grape pomace extracts total tannin content (TTC) was determined using the protein precipitation assay applied by Russo, Valentão, Andrade, Fernandez, and Milella (2015) (Russo et al., 2015). Data were expressed as mg of Tannic acid (TAE) equivalents per g of dried extract (mgTAE/g), considering the calibration curve of TAE.

2.4.1. Condensed tannins (CT)

Grape pomace extracts Condensed tannins were quantified following the protocol of Libutti et al. (Libutti et al., 2023). Data were expressed as mg of Catechin (CE) equivalents per g of dried extract (mgCE/g), considering the calibration curve of CE.

2.4.2. Hydrolyzable tannins (HT)

Hydrolyzable tannins were determined by applying the potassium iodate method of Russo et al. (2015) (Daniela Russo et al., 2015). Data were expressed as mg of Tannic acid (TAE) equivalents per g of dried extract (mgTAE/g), considering the calibration curve of TAE.

2.5. Total flavonoid content (TFC)

The total flavonoid content (TFC) was determined following the method of Russo et al. (2018) (Russo et al., 2018). Experiments were repeated three times. Results were reported as milligrams of Quercetin equivalents (QE) per gram of dried extract ± standard deviation (SD).

2.6. Anthocyanins (AN)

Anthocyanin content was quantified using the spectrophotometric assay described by Giusti and Wrolstad (2001). Extracts, at a concentration between 10 and 30 mg/mL, were solubilized under sonication (15 min) in acidified methanol (0.1 % *v/v* HCl) and diluted 1:5 using the same solvent. The absorbance was measured at 538 nm. Anthocyanins content was expressed as mg/g of malvidin 3-O-glucoside, considering the formula: $A*MW*5000\varepsilon*C$. Where: A is the absorbance spectrophotometrically measured at 538 nm; MW is the molecular weight of malvidin 3-*O*-glucoside (493.43 g/mol); 5000 is a conversion factor considering the sample dilutions; ε is the specific coefficient of absorptivity of malvidin 3-*O*-glucoside at 538 nm (29,500 $L \bullet M^{-1} \bullet cm^{-1}$); C is the concentration (mg/mL) of sample in the test solution.

2.7. Cell study

2.7.1. Cell lines and culture conditions

Human hepatoblastoma cells (HepG2) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 10 % fetal bovine serum (FBS), and 100 μg/mL streptomycin. Cells were stored in a humid atmosphere with 5 % CO_2 at 37 °C.

The immortalized human hepatocyte (IHH) cells were cultured in DMEM F-12 supplemented with 1 % of 100 IU/mL penicillin, 10 % FBS, 1 μM dexamethasone, 100 μg/mL streptomycin, and 10^{-12} M insulin. IHH cells were stored in a humid atmosphere with 5 % CO_2 at 37 °C.

2.7.2. Cell viability

Cell viability was determined using the MTT assay. HepG2 and IHH cells were cultivated in a 96-well culture plate until reaching a density of 1.5×10^4 and 1.0×10^4, respectively. Hence, cells were treated with grape pomace extracts (10–500 μg/mL) for 24 and 48 h. Each extract

was tested in triplicate, and the assay was performed using the method Faraone et al. (2022).

2.7.3. Measurement of intracellular reactive oxygen species (ROS)

The intracellular reactive oxygen species (ROS) were measured in HepG2 cells following the method of Faraone et al. (2022). Extracts were tested in triplicate.

2.8. LC-HRMS and LC-HRMS/MS analysis

Thermo LTQ Orbitrap XL high-resolution ESI mass spectrometer (Thermo Fisher, Waltham, MA, USA), coupled with a Thermo U3000 HPLC system (Thermo Fisher, Waltham, MA, USA) was used to conduct LC-HRMS and LC-HRMS/MS experiments. The HPLC setup featured a solvent reservoir, an in-line degasser, a binary pump, and a refrigerated autosampler. We utilized a Kinetex C18 column (50 × 2.10 mm, 5 μm particle size) maintained at room temperature. The elution process was executed at a flow rate of 200 μL/min with a gradient of H_2O (containing 0.1 % formic acid) and MeOH. The gradient program began with 10 % methanol for 3 min, followed by a linear increase to 100 % methanol over 30 min, and finished with 100 % methanol for 7 min. The injection volume was set at 5 μL. Mass spectra were acquired in positive ion mode, with the MS parameters set to a spray voltage of 4.8 kV, a capillary temperature of 285 °C, a sheath gas flow rate of 32 units N_2 (around 150 mL/min), and an auxiliary gas flow rate of 15 units N_2 (around 50 mL/min). Data collection employed the data-dependent acquisition (DDA) mode, selecting the five most intense ions from each full-scan mass spectrum for high-resolution tandem mass spectrometry (HRMS/MS) analysis. The DDA *m/z* range was 150 to 2000 amu.

HRMS/MS scans were carried out on the selected ions using collision-induced dissociation (CID) with an isolation width of 2.0, a normalized collision energy of 35, an activation Q of 0.250, and an activation time of 30 ms. Data were processed using Thermo Xcalibur software (version 2.2 SP1 build 48).

2.8.1. LC-HRMS/MS data processing and molecular networking

MZmine 2.53 (Pluskal, Castillo, Villar-Briones, & Oresic, 2010) software was used to import and pre-process raw data files maintaining a noise level of 10,000 for mass detection performed on both raw data and centroided masses at levels 1 and 2. ADAP module with a minimum height of 50,000 and an *m/z* tolerance of 0.01 (or 20 ppm) was performed to construct chromatograms. Peak alignment was conducted using the Join aligner algorithm with the following parameters: an *m/z* tolerance of 0.005 (or 5 ppm) and an absolute retention time (RT) tolerance of 0.5 min. A .mgf file was created from feature-based data for Global Natural Products Social Molecular Networking (GNPS), and chromatographic data, including retention times, peak areas, and peak heights, were exported to a .csv file.

A Feature-Based Molecular Network (Nothias et al., 2020) was created using the GNPS online platform (UCSD Computational Mass Spectrometry Website (Wang et al., 2016) (https://gnps.ucsd.edu). The parameters for network generation included a precursor ion mass tolerance and MS/MS fragment ion tolerance of 0.02 Da. To be accepted, spectra needed a cosine score greater than 0.4 and at least 5 matched peaks. Spectra were only retained if nodes appeared in each other's top 10 most similar nodes. The criteria for the GNPS library search were a cosine score of 0.6 and a minimum of 5 matched peaks. The molecular network visualization was performed using Cytoscape (Shannon et al., 2003) software (version 3.10.1).

2.9. Statistical analysis

The experiments were performed in triplicates, and results were reported as mean ± standard deviation (SD). Statistical analysis was evaluated with GraphPad Prism 5 (La Jolla, CA 92093–0608, United States) using one-way analysis of variance (ANOVA) to determine any significant differences between the samples, with a confidence level of 95 %. Tukey's test matched the means and identified any significant differences, with a significance level set at $p < 0.05$.

Table 1
Antioxidant activity evaluation.

	Yield (%)	TPC mgGAE/g	DPPH mgTE/g	FRAP mgTE/g
Sample	mean ± dev.st	mean ± dev.st	mean ± dev.st	mean ± dev.st
B ASE	7.56 ± 0.45 ^{e,f}	327.49 ± 19.60 ^{a}	858.84 ± 43.05 ^{b}	915.69 ± 47.22 ^{a}
T ASE	9.38 ± 0.92 ^{c,d,e}	258.72 ± 20.13 ^{b,c}	719.57 ± 48.27 ^{c,d}	739.44 ± 29.27 ^{b,c}
CNS ASE	10.37 ± 1.33 ^{b,c,d}	216.02 ± 19.69 ^{c,d}	797.55 ± 65.15 ^{b,c}	825.72 ± 83.53 ^{a,b}
B MW	8.72 ± 0.28 ^{d,e,f}	283.70 ± 3.27 ^{a,b}	695.39 ± 5.50 ^{c,d}	616.79 ± 31.52 ^{c,d,e}
T MW	13.36 ± 1.01 ^{a}	246.39 ± 21.41 ^{b,c}	988.88 ± 53.81 ^{a}	342.93 ± 32.55 ^{g,h}
CNS MW	11.42 ± 1.42 ^{a,b,c,d}	254.58 ± 19.22 ^{b,c}	684.53 ± 43.10 ^{d}	495.85 ± 39.77 ^{e,f}
B US	9.47 ± 1.22 ^{b,c,d,e}	192.66 ± 13.15 ^{d}	455.92 ± 36.75 ^{e,f,g}	461.60 ± 45.30 ^{f,g}
T US	11.59 ± 0.58 ^{a,b,c}	184.99 ± 16.43 ^{d}	546.97 ± 28.98 ^{e}	351.40 ± 14.68 ^{g,h}
CNS US	12.20 ± 1.02 ^{a,b}	191.61 ± 18.83 ^{d}	231.52 ± 8.93 ^{h}	316.71 ± 13.98 ^{h}
B DIG	7.38 ± 0.19 ^{e,f}	224.11 ± 7.89 ^{c,d}	557.67 ± 5.92 ^{e}	703.43 ± 62.28 ^{b,c}
T DIG	11.51 ± 1.30 ^{a,b,c}	221.78 ± 9.81 ^{c,d}	492.83 ± 22.53 ^{e,f}	560.09 ± 12.61 ^{d,e,f}
CNS DIG	11.45 ± 1.01 ^{a,b,c,d}	217.90 ± 17.03 ^{c,d}	433.89 ± 19.35 ^{f,g}	653.60 ± 63.23 ^{c,d}
B MAC	6.10 ± 0.85 ^{f}	242.70 ± 18.68 ^{b,c}	559.72 ± 41.64 ^{e}	515.85 ± 43.52 ^{e,f}
T MAC	9.89 ± 0.20 ^{b,c,d,e}	229.56 ± 14.48 ^{c,d}	351.98 ± 1.93 ^{g}	733.69 ± 63.06 ^{b,c}
CNS MAC	9.02 ± 0.53 ^{c,d,e}	220.11 ± 15.47 ^{c,d}	497.74 ± 44.66 ^{e,f}	465.87 ± 21.70 ^{f,g}

ASE, accelerated solvent extraction; MW, microwaves; DIG, digestion; MAC, maceration; US, ultrasounds. Grape pomace from Aglianico Grape of Barile (B), Torrecuso (T), and Cantine del Notaio (CNS). The results are reported as mean ± standard deviation; different letters (a-h) indicate statistically significant differences ($p < 0.05$).

3. Results and discussions

3.1. Extractive yields and antioxidant activity evaluation

In the case of the present investigation, by using MAC, DIG, ASE, MW, and US and environmentally friendly solvents like 50 % ethanol/water, it was obtained extractive yields ranging between 6.10 % ± 0.85 and 13.36 % ± 1.01 (Table 1). Hence, the antioxidant activity of each extract obtained was evaluated. Free radicals represent a group of harmful molecules normally produced during the human body's cell metabolism. However, exposure to environmental pollutants, cigarette smoking, ultraviolet radiation, or stress may enhance the level of free radicals produced, leading to the incapacity of the human antioxidant defense to counteract them. In this condition, the excess of free radicals can cause harm to enzymes, DNA, and membranes with the consequent outbreak of several human diseases such as cancer, neurodegenerative disease, metabolic syndromes, cardiovascular problems, and others (Akbari, Baghaei-Yazdi, Bahmaie, & Mahdavi Abhari, 2022). Implementing the assumption of exogenous antioxidant substances by including fruit and vegetables in the diet is encouraged to avoid this condition. Over the last decade, numerous investigations have demonstrated that agro-industrial by-products represent promising sources of high-value antioxidant molecules (Reguengo, Salgaço, Sivieri, & Maróstica Júnior, 2022). Due to the high levels of phenolic molecules, GP represents a suitable by-product that is usable as a source of health-

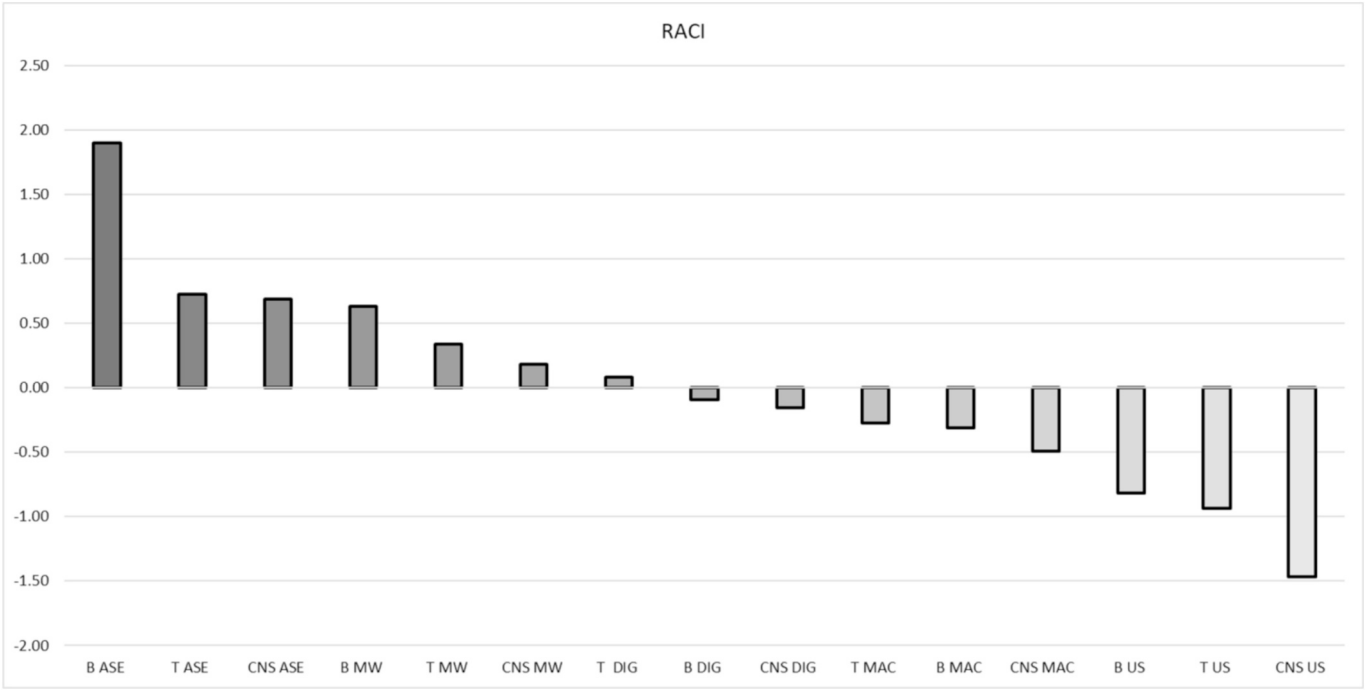

Fig. 1. RACI (Relative capacity index) of extract made with different extraction methods (DIG, digestion; MAC, maceration; MW, microwaves; US, ultrasounds; ASE, accelerated solvent extraction) GP from Aglianico Grape of Cantine del Notaio (CNS), Torrecuso (T), and Barile (B).

promoting antioxidants. For this reason, it was decided to investigate the antioxidant power of the three different Aglianico GP using three different tests: DPPH, TPC, and FRAP. It is known that evaluating an extract's antioxidant activity cannot be achieved comprehensively through a single test. This is because antioxidants represent a diverse group of molecules, each capable of neutralizing specific radicals or oxidants, depending on their distinct chemical structures. It is essential to employ multiple techniques to fully understand an extract's various antioxidant properties (De Rosa et al., 2024). Results from the antioxidant activity evaluation are listed in Table 1. As possible to observe, the highest antioxidant values were obtained for ASE, which allowed obtaining a higher TPC and DPPH value compared to those on Cabernet Sauvignon GP, where the application of a Pressurized Hot Water Extraction results in a TPC value of 4.2 mgGAE/g and 4.1 mgGAE/g for fermented and unfermented grape pomace, respectively, and in a DPPH value of 184 mg TE/g for fermented pomace (Vergara-Salinas et al., 2013). The efficacy of hydroalcoholic extraction compared to the aqueous one is confirmed by previous investigations on GP in which it was seen that mixtures of methanol or ethanol with water are more effective than water used as a single solvent. This is in line with the nature of alcohol since they are less polar than water and are then more effective in seed and cell wall degradation with the consequent higher release of active metabolites (Lapornik, Prošek, & Golc Wondra, 2005). Results for the TPC and DPPH values achieved with the US extraction are higher than those obtained from another investigation on Lacrima di Morro d'Alba GP. In this case, in fact, 41.2–45.9 mgGAE/g and 82.8–138.2 mgTE/g employing 70 % EtOH/ H_2O acidified with HCl (0.1 %) were obtained for TPC and DPPH, respectively (Abouelenein et al., 2023). On the other hand, data obtained in the present investigation are comparable to those on US extraction of Aglianico GP, where a total phenolic content of 190 mgGAE/g using 50 %MeOH/H_2O as an extractive solvent was attained (Crescente et al., 2023). This evidence indicates that ethanol may be a viable eco-friendly and safe alternative to methanol in extracting specialized metabolites from Aglianico pomace.

The Relative Antioxidant Capacity Index (RACI) was calculated to compare the results obtained from DPPH, TPC, and FRAP assays with a view to identifying the most effective extraction method (Fig. 1).

The RACI allows confirmation of the effectiveness of the hydroalcoholic ASE extraction method and indicates the extract from Barile GP as the most active, followed by Cantine del Notaio and Torrecuso GP. The observed difference in the antioxidant activity of the different GP varieties confirms previous observations since it was seen that the growing region of the wine, genotype, weather, and winemaking techniques may influence the phenolic amount and the antioxidant activity of wine by-products (Di Stefano et al., 2022).

Considering the RACI, it was decided to focus on ASE, as an unconventional extraction tecnique, and DIG, as a conventional extractive method. The extracts made by DIG are in fact the most active compared to those obtained with MAC, the other conventional extraction technique.

3.2. *Phytochemical analysis: In vitro spectrophotometric assays*

An important quality factor for red wines is represented by polyphenols, which are highly related to wine sensory descriptors like structures, astringency, and bitterness. Amidst the by-products produced during winemaking, the pomace retains high total polyphenol content depending on the grape variety or winemaking techniques. A great deal of work has been devoted to developing analytical methods for determining grape polyphenolic composition with a view to planning interventions, from selecting the harvest date to controlling winemaking and wine aging processes (Bosso, Guaita, & Petrozziello, 2016). However, only a few investigations were performed on evaluating Aglianico Grape pomaces, and no extraction methods have been defined. Hence, in this section, the effect of two different extraction techniques, DIG (as a conventional extractive method) and ASE (as an unconventional extractive technique), coupled with hydroalcoholic solvent, will be compared based on the recovery of tannins, flavonoids, and anthocyanins from different Aglianico grape pomace.

3.2.1. *Tannins*

The name Tannin is generally used to describe a polyphenolic nature complex biomolecule that has a sufficient number of hydroxyl and other

Table 2
Phytochemical analysis.

SAMPLE	TFC mgQE/g	AN mgMA/g	TTC mgTAE/g	HT mgTAE/g	CT mgCE/g
B ASE	555.38 ± 40.18 ^{b}	13.38 ± 0.57 ^{c}	2253.62 ± 112.28 ^{a,b,c}	971.16 ± 73.23 ^{a,b,c}	238.86 ± 14.73 ^{a}
T ASE	709.02 ± 53.31 ^{a}	13.09 ± 0.75 ^{c}	2209.71 ± 127.55 ^{a,b,c}	780.3 ± 72.56 ^{c}	220.33 ± 7.46 ^{a,b}
CNS ASE	405.88 ± 30.63 ^{c}	14.04 ± 0.92 ^{b,c}	2140.93 ± 206.23 ^{b,c}	999.21 ± 52.66 ^{a,b}	212.45 ± 15.14 ^{a,b}
B DIG	801.34 ± 54.18 ^{a}	17.08 ± 0.76 ^{a}	2676.37 ± 223.86 ^{a}	843.43 ± 79.02 ^{b,c}	133.46 ± 10.35 ^{d}
T DIG	810.81 ± 51.61 ^{a}	15.71 ± 1.00 ^{a,b}	1912.53 ± 128.95 ^{c}	1005.15 ± 37.08 ^{a,b}	188.62 ± 17.3 ^{b,c}
CNS DIG	437.01 ± 35.75 ^{b,c}	14.66 ± 0.70 ^{b,c}	2548.48 ± 227.03 ^{a,b}	1136.05 ± 107.34 ^{a}	152.34 ± 13.22 ^{c,d}

DIG, digestion; ASE, accelerated solvent extraction. Aglianico Grape of Torrecuso (T), Barile (B), and Cantine del Notaio (CNS); TFC, total flavonoid content; AN, anthocyanins; TTC, total tannins content; HT, hydrolizable tannins; CT, condensed tannins. The results are reported as mean ± standard deviation; different letters (a-d) indicate statistically significant differences ($p < 0.05$).

groups, such as carboxyls, to form stable complexes with a wide variety of macromolecules (Das, Islam, Faruk, Ashaduzzaman, & Dungani, 2020). Tannins are secondary plant phenolic compounds commonly grouped into hydrolyzable and condensed tannins. Hydrolyzed tannins are formed by a carbohydrate core, commonly glucose, esterified with hexahydroxydiphenic acid (ellagitannins) and gallic acid (gallotannins). Condensed tannins are, instead, polymers or oligomers of flavonoid units joined by carbon-carbon bonds. Condensed tannins have a broader range of molecular weights than hydrolyzable tannins, from 500 to over 20,000, and have the capacity to form polymeric materials by reacting with aldehydes (Das et al., 2020). In the context of the present investigation, the content of total tannins in Aglianico grapes pomaces varied between 1912.53 ± 128.95 and 2676.37 ± 223.86 mgTAE/g (Table 2). Comparisons can not be made with data present in literature due to the use of different measurement units; however, by analyzing individually the amount of condensed and hydrolyzable tannins, it was seen that the second one overcame the content of the first one. This is in contrast with data found in literature, where condensed tannins are the most abundant in grape pomace (Bosso et al., 2016). However, the obtained results could be explained by the unequal partition of condensed and hydrolyzable tannins within the grape, with condensed tannins being more prevalent in the seeds and hydrolyzable tannins more concentrated in the skins (Watrelot & Norton, 2020). It is known that the tannins present in *Vitis vinifera* and other *Vitis* species differ greatly in their composition and concentration in their skins compared to their seeds (Watrelot & Norton, 2020). Therefore, the differences found in the present investigation can be related to the different seed-to-skin ratios in the samples analyzed. Hydroalcoholic DIG and ASE methods yield similar amounts of tannin, indicating that both extraction techniques are effective for obtaining these specialized metabolites.

3.2.2. Flavonoids and anthocyanins

Nearly 6000 flavonoid molecules have been reported in plants, classifying flavonoids as a significant family of secondary metabolites representing 13–30 % of the total phenolic content of red grapes. These compounds are predominantly located in the outer cells of the epidermal cells (the skin of the grape), making the grape pomace a valuable source of these secondary metabolites (Georgiev, Ananga, & Tsolova, 2014). The most abundant flavonoids to be found in the grape are anthocyanins, which are present only in red pomace. They are mainly concentrated in the berry's skin, but in some cultivars, called "*teinturier*" (or "colored"), the anthocyanin pigments are located in the flesh of the berry (Georgiev et al., 2014). Furthermore, each variety of grape possesses a distinctive profile of anthocyanins; for instance, European grapes exclusively produce anthocyanidins 3-*O*-monoglucoside, while Muscadine grapes contain only anthocyanidins 3,5-*O*-diglucosides (Zhu, Zhang, & Lu, 2012).

In the present investigation, the amount of flavonoids varied from 405.88 ± 30.63 to 810.81 ± 51.61 mgQE/g, while that of anthocyanins ranged between 13.09 ± 0.75 and 17.08 ± 0.76 mgMA/g (Table 2). The greatest quantity of anthocyanins was achieved in Torrecuso and Barile grape pomace varieties subjected to hydroalcoholic DIG, obtaining higher values than those of Salento grape pomace (from 5.3 ± 1.7 to 10.3 ± 1.4 mgMA/g) (Negro et al., 2021). However, these differences may be related to berry development since a close correlation has been found between anthocyanin biosynthesis and the degree of berry ripeness. Anthocyanin biosynthesis indeed begins at "*veraison*", when the biosynthesis of proanthocyanidins is completed and attains its maximal level at berry "*ripeness*" (Zhang, Che, Pan, Li, & Duan, 2013). Noteworthy is also the different extractive processes used since Negro et al. (2021) (Negro et al., 2021) employed maceration while, in the present study, it was used digestion at 60 °C, indicating the importance of temperature for flavonoid extraction. Corroborating this assumption, results from a previous investigation showed that the highest levels of extracted flavonoids were achieved at high temperatures (60–90 °C) (Tan, Parks, Stathopoulos, & Roach, 2014).

3.3. Metabolic profile

Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was used to analyze extract samples. Dereplication of HRMS data was performed using molecular networking, as detailed in previous studies (Esposito et al., 2023; Teta et al., 2022). In particular, we focused on the most promising extracts in antioxidant assays (B ASE, T ASE, CNS ASE, B DIG, T DIG, CNS DIG). The resultant network comprised 238 nodes, 27 of which were organized into 9 clusters and 211 self-loop (Fig. 2). Each node is shown as a pie chart representing the different extracts analyzed (pink, CNS DIG; green, B DIG; blue, T DIG;

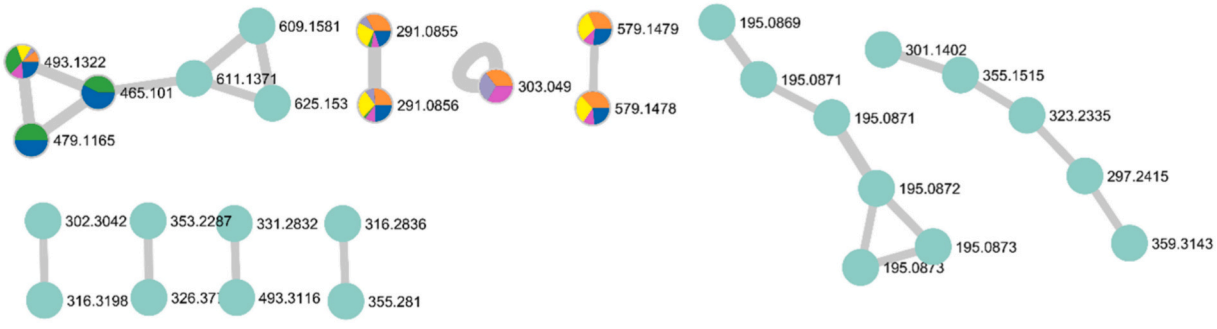

Fig. 2. Portion of molecular network of Aglianico grape pomace extracts. The nodes are represented as a pie chart responding to the compounds' source extracts. (pink, CNS DIG; green, B DIG; blue, T DIG; orange, B ASE; purple, CNS ASE; yellow, T ASE). Nodes in light green represent compounds that are not identified. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

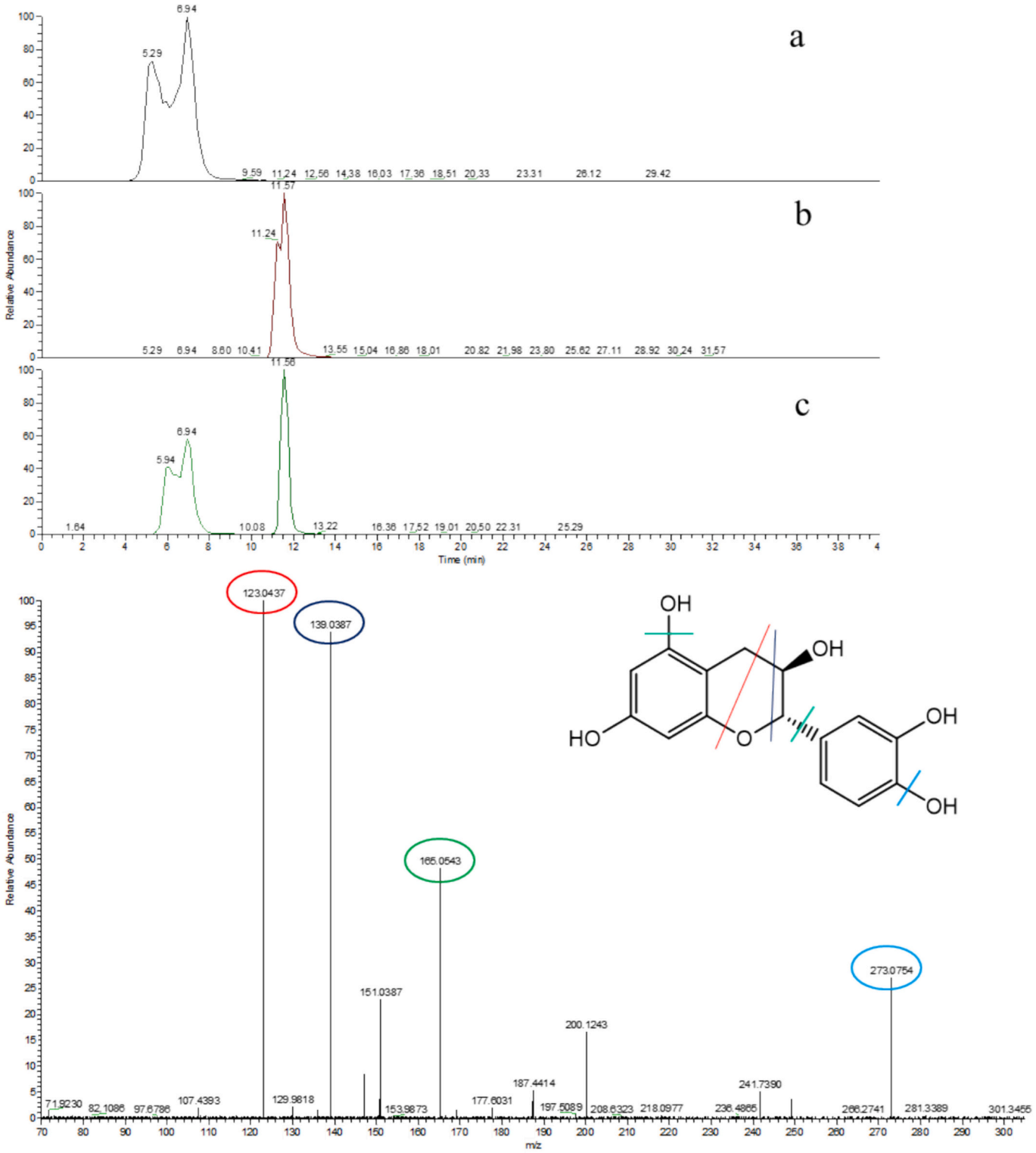

Fig. 3. (a) extracted-ion chromatograms at *m/z* 291.0856 of authentic catechin, (b) extracted-ion chromatograms at *m/z* 291.0856 of authentic epicatechin, (c) extracted-ion chromatograms at *m/z* 291.0856 of catechin/epicatechin from B ASE extracts. Positive-ion high-resolution MS/MS spectrum of catechin, parent ion at *m/z* 291.0.

orange, B ASE; purple, CNS ASE; yellow, T ASE). The molecular network unveiled an array of compounds showing chemical affinities with anthocyanins, along with peonidin or other glycosylated versions of malvidin or petunidin. The correspondence between the node with *m/z* 291.0856 and catechin and epicatechin ($C_{15}H_{15}O_6^+$) in the GNPS library supported the identification of the cluster recorded with molecules belonging to the catechin family.

The analysis of the LC-HRMS spectra of extracts compared with that of catechin and epicatechin standards confirmed this identification by showing two peaks at RT 6.94 and 11.56 for the two stereoisomers. In addition, the HR-MS/MS spectrum showed the diagnostic fragments ions at *m/z* 273.0749 ($C_{15}H_{13}O_5^+$), *m/z* 165.0541 ($C_9H_9O_3^+$), *m/z*

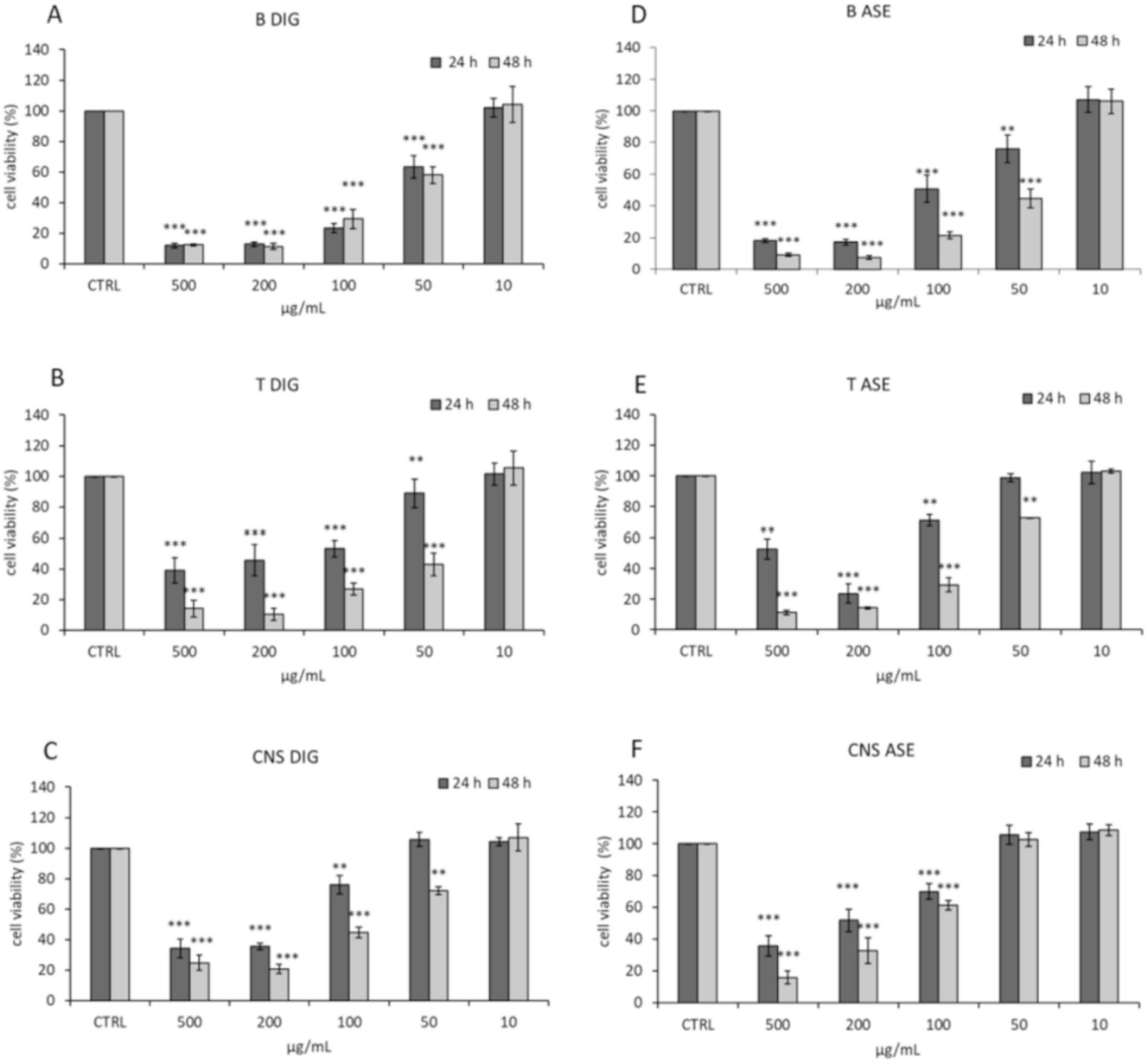

Fig. 4. Cell viability on immortalized liver cancer cell line HepG2 treated with different concentrations (10–500 µg/mL) Aglianico Grape of **A-D**) Barile (B), **B-E**) Torrecuso (T), and **C—F**) Cantine del Notaio (CNS) hydroalcolic extracts. DIG, digestion; ASE, accelerated solvent extraction.

139.0390 ($C_7H_7O_3^+$) and *m/z* 123.0441 ($C_7H_7O_2^+$) (Fig. 3). The node at m/z 303.049 was identified as quercetin ($C_{15}H_{11}O_7^+$) on analyzing the fragmentation patterns showing diagnostic fragment ions at *m/z* 285.0391 ($C_{15}H_9O_6^+$), *m/z* 257.0441 ($C_{14}H_9O_5^+$), *m/z* 229.0491 ($C_{13}H_9O_4^+$), *m/z* 165.0180 ($C_8H_5O_4^+$) (Fig. S1). It is reported in the literature that catechin and epicatechin are the primary components in the seeds, whereas flavonoids are dominant in the peel extracts. This difference in extract composition is attributed to the varying seed-to-peel ratios in the pomace samples. The elevated levels of catechin in the B and T samples likely result from a higher seed content in the pomace. At the same time, quercetin is predominantly found in the CNS samples, which are characterized by a higher peel content.

Among the anthocyanins, our analysis revealed the presence of a cluster containing petunidin 3-*O*-glucoside corresponding to the node at *m/z* 479.1165. The fragmentation pattern provided clear evidence of the loss of the sugar group with a fragment ion at *m/z* 317.0648 corresponding to the molecular formula $C_{16}H_{13}O_7^+$ and the fragment ion at *m/z* 303.0492 resulted from the loss of a further methyl group (Fig. S2). This cluster includes also malvidin-3-O-glucoside (node at *m/z* 493.1322) and delphinidin-3-O-glucoside (*m/z* 465.101), both of which showed loss of sugar in their fragmentation pattern (Figs. S3 and S4). The other three nodes belonging to the cluster were not identified. The present analysis of the Aglianico pomace extracts thus shows similar amounts of catechins and anthocyanins and no stilbenes. Analyzing the LC-HR/MS-MS data referred to the node at *m/z* 579.1478 allowed identifying procyanidin B2 ($C_{30}H_{27}O_{12}^+$) presenting fragment ions at *m/z* 427.1017 ($C_{22}H_{19}O_9^+$), *m/z* 409.0912($C_{22}H_{17}O_8^+$), *m/z* 291.0859 ($C_{15}H_{15}O_6^+$) (Fig. S5). The results obtained align with existing literature, recognizing that physiological differences in red wine pomace can arise from variations in cultivation and vinification procedures. The presence of stilbenes, including the very low levels of resveratrol, highly depends on grape varieties. This low resveratrol content can be attributed to the role of specialized metabolites as defense agents; in the absence of infections or physiological stress, resveratrol levels remain low. Additionally, resveratrol may transfer into the wine, falling below detection limits in the pomace.

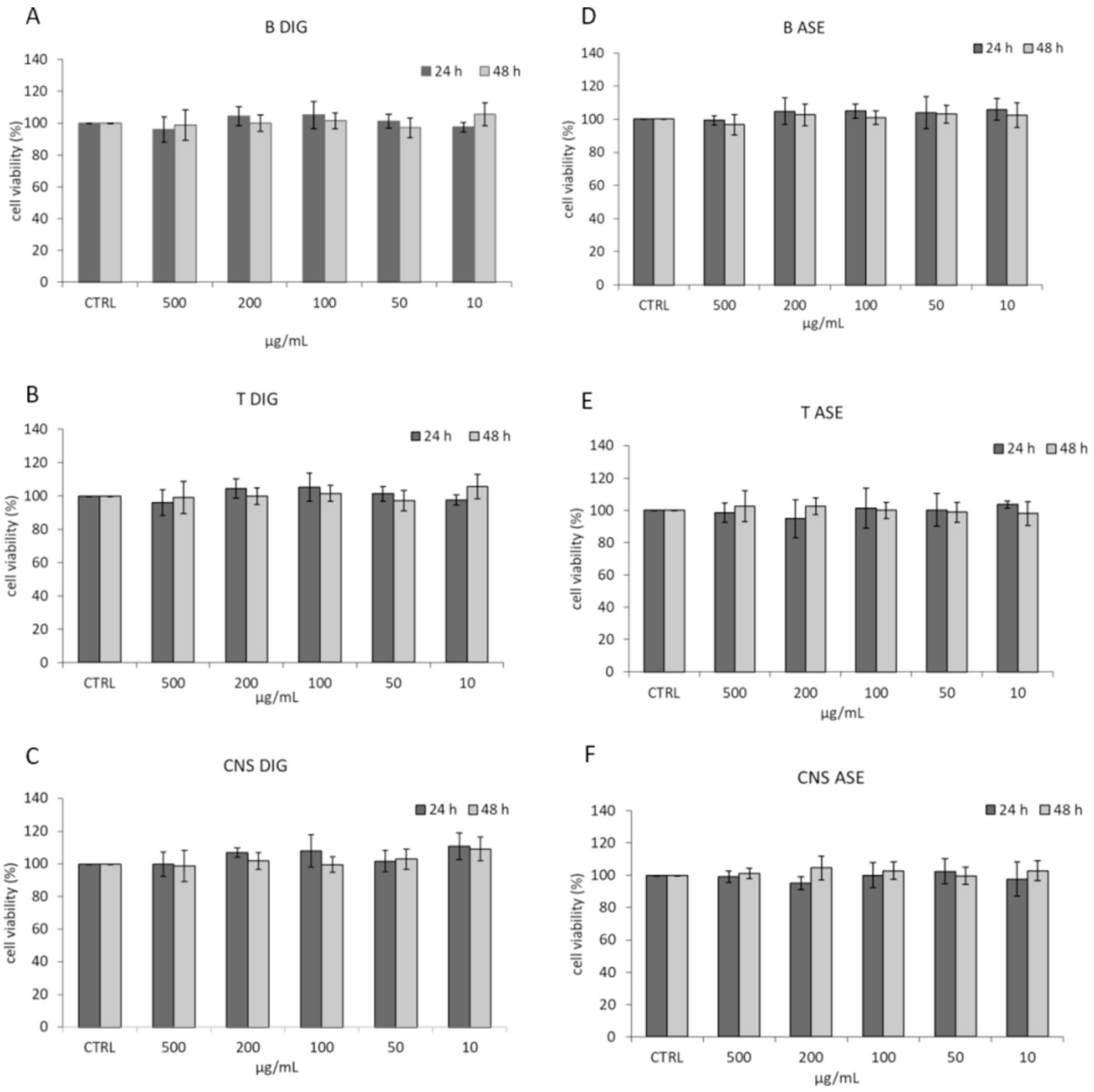

Fig. 5. Cell viability on immortalized human hepatocytes IHH treated with different concentrations (10–500 µg/mL) Aglianico Grape of **A-D**) Barile (B), **B-E**) Torrecuso (T), and **C—F**) Cantine del Notaio (CNS) hydroalcolic extracts. DIG, digestion; ASE, accelerated solvent extraction.

Consequently, it is possible to conclude that the Aglianico variety is deficient in phenolic acids despite being rich in terpenes and anthocyanins, which offer promising health benefits. This study not only provides the first comprehensive analysis of the chemical composition of Aglianico pomace but also identifies previously unreported compounds with potential antioxidant and chemopreventive properties. Further analyses are necessary to purify and chemically characterize these unknown compounds.

3.4. In vitro cell-based investigations

3.4.1. Anti-proliferative activity evaluation

A growing number of investigations identified specialized molecules from plant and their by-products as agents able to induce cell apoptosis, thereby playing a crucial role in clearing mutated hyperproliferating cells. It was demonstrated that phenols may exert anti-proliferative and antineoplastic activities by influencing differentiation, proliferation, and apoptosis in various cancer cells (Caponio et al., 2022). Specifically, active compounds from grape pomace, like anthocyanidins and flavonoids, demonstrated proapoptotic and growth inhibition effects on different cancer cell lines. However, as far as is known, no study reported the potential activity of Aglianico grape pomace on hepatocarcinoma cell lines. For this reason, once the extracts' antioxidant activity and presence of tannins, flavonoids, and anthocyanins were validated, the Aglianico grape pomace activity was investigated on either IHH hepatic cells or HepG2 cancer cells.

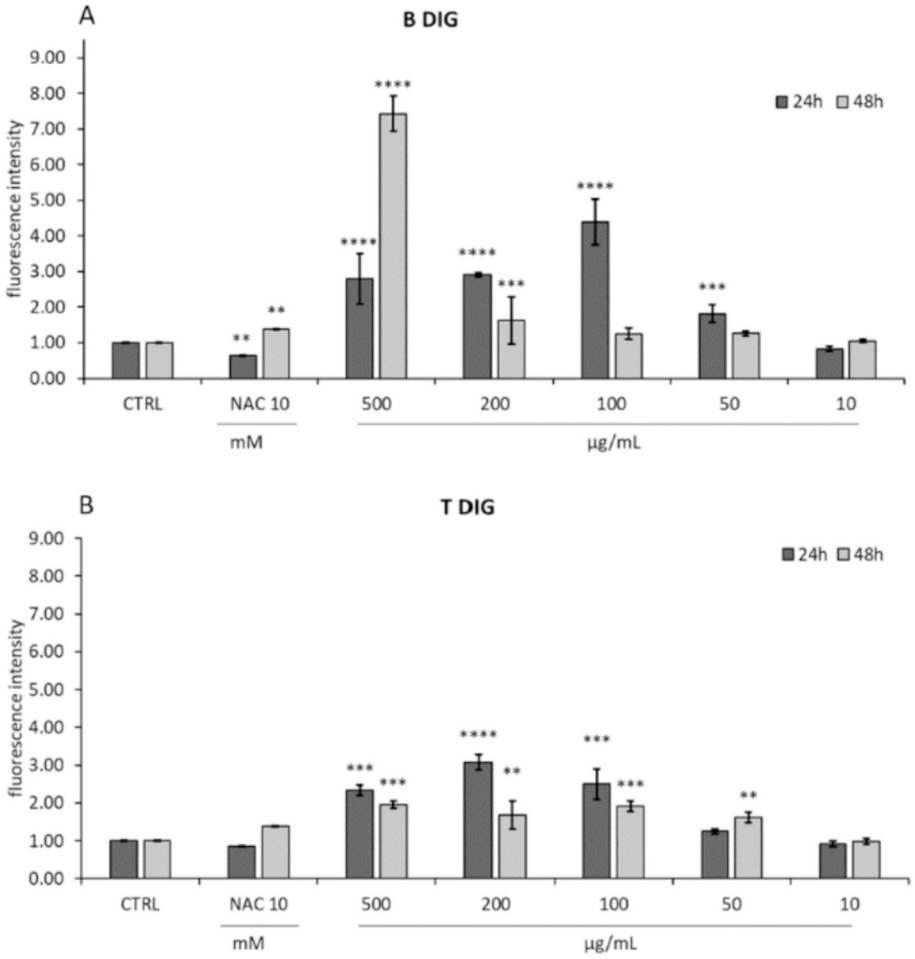

Fig. 6. ROS levels were determined in HepG2 cells treated for 24 h and 48 h with **A**) Barile (B) and **B**) Torrecuso (T) extract obtained by hydroalcoholic digestion (DIG). Results are presented as the mean ± standard deviation of three independent experiments ($n = 3$). ** $p < 0.01$, ***$p < 0.001$, *vs* Control (CTRL). NAC, acetyl-L-cysteine.

All of the extracts examined reduced the viability of HepG2 cells in a dose-dependent manner at 24 and 48 h (Fig. 4). The data expressed in terms of IC_{50} are shown in Table S1. B DIG and T DIG were the most active extracts after 24 h (IC_{50} of 58.72 ± 5.88 µg/mL and 98.75 ± 8.27 µg/mL, respectively) and 48 h (IC_{50} of 49.40 ± 6.20 µg/mL and 63.04 ± 4.54 µg/mL, respectively), thus confirming spectrophotometric assay since these extracts showed the highest amount of flavonoids and anthocyanins. A previous investigation corroborated these results, showing that anthocyanin-rich Pinot noir grape pomace extract exhibited a cytotoxic activity on HepG2 cells, even if with a greater IC_{50} (200 µg/mL) than Aglianico grape pomace (De Sales et al., 2018). This cytotoxic activity may be due to the ability of Aglianico grape pomace phenols to affect proliferation and apoptosis in cancer cells. A previous investigation on gastrointestinal digested Aglianico grape pomace extract, indeed, demonstrated that this by-product might determine a significant increase of the proapoptotic marker Bax and reduction in the anti-apoptotic marker Bcl-2 in HT29 and SW480 cells, thereby inducing the mitochondrial apoptotic pathway (Caponio et al., 2022). Supporting this data, there are also those from another study on HT29, MCF-7, and PC-9 cells treated with proanthocyanidins, in which a significant decrease in Bcl-2 and an increase in Bax was observed (Albogami, 2020). Likewise, it was demonstrated that the muscadine grape "noble" pomace extract rich in anthocyanins can reduce the viability of human metastatic adenocarcinoma MCF-7 cells by forcing the cycle arrest in the S phase and inducing apoptosis (Luo et al., 2017).

Aglianico grape pomace extracts were also evaluated for their effect on nontumor cells IHH, showing no cytotoxic activity (Fig. 5). This is an essential aspect and reflects the nature of phenols, which can exert a differential activity by targeting cancer cells but not normal ones (Batra & Sharma, 2013; Rengarajan & Yaacob, 2016). As in the present investigation, a recent study indeed demonstrated that grape pomace extract rich in polyphenols, mainly anthocyanins, was cytotoxic in hepatocarcinoma cells but not in human non-cancer fibroblasts (De Sales et al., 2018).

Therefore, based on the results obtained, it can be concluded that Barile and Torrecuso extracts obtained with DIG have the highest antiproliferative activity on HepG2. This enhanced activity may be ascribable to the higher flavonoid and tannin content present in these extracts, indicating that the temperature used in the extraction process plays a crucial role in obtaining these metabolites. Previous studies have indeed shown that the optimal extraction temperature for flavonoids is between 60 and 90 °C. In contrast, using ASE at temperatures of 120 °C might lead to degradation or alteration of the chemical structure of these compounds, ultimately reducing their activity (Tan et al., 2014). DIG is a particularly advantageous extraction method when thermolabile or bioactive compounds are to be extracted, ensuring a high-quality product at a lower cost. Furthermore, DIG is generally preferred for industrial-scale extraction because it is cheaper, simpler, safer, and more sustainable than ASE. Otherwise, ASE remains useful for specific laboratory applications, but its scalability and cost challenges make it less suitable for high-volume industrial applications.

3.4.2. Effect on intracellular ROS

It is known that phenols have distinct mechanisms for regulating the

cells' redox potential depending on their antioxidant and pro-oxidant activities. When exerting antioxidant action, phenols act through three different mechanisms: induction of the antioxidant defense system, scavenging oxidants, and transition metals complexation. In contrast, polyphenols' pro-oxidant activities are mainly investigated for use in the anti-cancer fields and are supported by their capacity to produce phenoxyl radicals or ROS upon exposure to transition metals (Dzah, Zhang, Gobe, Asante-Donyinah, & Duan, 2024). Thanks to this double activity, phenols may induce healthy cells' mitogenesis or aberrant cells' mitophagy by activating or inactivating the PKD/NF-kB pathway. This effect depends on the cellular environment and extract dosage, particularly under conditions of oxidative stress (Dzah et al., 2024). Considering this background, the most cytotoxic Aglianico grape pomace extracts (B DIG and T DIG) were investigated for their effect on intracellular ROS in hepatocarcinoma HepG2 cells. Therefore, HepG2 cells were exposed to B DIG and T DIG extracts (10–500 μg/mL) for 24 h and 48 h with different concentrations, observing a dose-dependent increase in ROS levels (Fig. 6). B DIG extract, in particular, showed the greatest pro-oxidant effect. This increase in oxidative stress might be one of the possible mechanisms that underwent the demonstrated anti-proliferative effects. These findings align with previous investigations demonstrating that either extracts from red (Grace Nirmala, Evangeline Celsia, Swaminathan, Narendhirakannan, & Chatterjee, 2018) or white (León-González, Jara-Palacios, Abbas, Heredia, & Schini-Kerth, 2017) grape pomace induce cytotoxic effects by generating ROS, leading to caspase activation and apoptosis (Choi et al., 2016). The pro-oxidant effect of grape pomace may be related to the presence of terpenes and flavonoids since they can trigger oxidative stress and DNA damage in cancer cells, thereby activating the cells' apoptosis machinery (Machado et al., 2024; Wróblewska-Łuczka, Cabaj, Bargieł, & Łuszczki, 2023).

4. Conclusions

This study explored the potential of three different Aglianico grape pomace (GP), a by-product of the wine supply chain, as a sustainable source of bioactive compounds with antioxidant and anti-proliferative properties. Using five eco-friendly extraction methods (MAC, DIG, MW, US, ASE), it was demonstrated that the choice of extraction technique significantly influences the bioactivity of the recovered compounds. Our results demonstrated that DIG emerged as the most effective technique, particularly for Barile GP extracts, which exhibited high cytotoxicity against HepG2 cancer cells, exerting a pro-oxidant effect, sparing non-tumor hepatic cells. These findings suggest that DIG could be a cost-effective and industrially scalable method for producing high-value bioactive extracts. The major strength of this work lies in its integrative approach, combining eco-friendly extraction methods with advanced analytical techniques such as HRMS/MS and molecular networking. This latter allowed detailed metabolic profiling of Aglianico GP extracts, identifying known and unknown compounds with promising antioxidant and chemopreventive properties. Additionally, the study highlights the potential of these extracts for their application in nutraceutical and pharmaceutical fields, contributing to the valorization of agro-industrial by-products. Future research will be focused on expanding the biological evaluation on different *in vitro/in vivo* models and GP varieties to get additional findings into viable health-promoting sustainable extract applications.

CRediT authorship contribution statement

Maria Ponticelli: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Germana Esposito:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Fabiana Labanca:** Writing – original draft, Methodology, Investigation, Data curation. **Paolo Scognamiglio:** Writing – review & editing. **Chiara Sinisgalli:** Methodology, Investigation. **Luigi Milella:** Writing – review & editing, Validation, Supervision, Resources, Project administration. **Immacolata Faraone:** Writing – review & editing, Supervision. **Valeria Costantino:** Writing – review & editing, Validation, Supervision, Resources.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.142573.

Data availability

Data will be made available on request.

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