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Oleanolic acid: A promising antidiabetic metabolite detected in Aglianico grape pomace

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ABSTRACT

Grape pomace, a bulky component of winery waste, is a source of healthy compounds. So far, scientific research has mainly focused on its polyphenol content, but given the impressive number of bioactivities shown by grape pomace, it is not unlikely that, besides polyphenols, additional metabolites, so far undetected, may be involved. In order to verify such hypothesis, an in-depth chemical analysis of Aglianico (*Vitis vinifera*) grape pomace was conducted by NMR and LC-MS/MS. In addition to a number of polyphenols, a remarkable concentration of oleanolic acid (0.45 mg/g - fresh weight) was determined in the analyzed material. Oleanolic acid is a natural triterpenoid showing many bioactivities including antitumor, anti-inflammatory, antibiotic and antiviral properties. Also, it was proven a potential antidiabetic molecule in Type1 Diabetes rats. Hence, its influence on the mitochondrial and glucose uptake activities of C2C12 myoblast was here assessed, thus supporting oleanolic acid as a promising antidiabetic metabolite.

1. Introduction

Annually over 70 million tons of grapes are produced across the planet and approximately 70% of the total grape production is employed for winemaking (FAO, 2019; García-Lomillo & González-SanJosé, 2017). In major wine producing countries, impressive amounts of waste are generated by wineries, which cause environmental concerns such as air pollution, leaching and odors (Drevelegka & Goula 2020). It has been estimated that some 20–25% of the grape weight account for the main winery by-product usually referred to as grape or wine pomace (or marc). Grape pomace is constituted by residual pulp, pressed skins, seeds, pieces of stalks and yeast cells used in the fermentation phases of winemaking. This material has long been regarded as an unvaluable waste and a heavy economic and environmental burden to deal with. Therefore, over the past decades some possible reuses of pomaces have been proposed, in order to valorize it and derive economic benefits. Among major alternatives, distillation procedures have long been used to obtain distilled spirits, liquors and liqueurs (Gonzalez-SanJose, 2014). Alternatively, pomaces have been exploited to produce feed for farm animals or even fertilizing material to improve the nitrogen and mineral concentrations in vineyard soils (Soceanu et al., 2021). However, in either case there are disadvantages, since grape pomaces also contain antinutritive molecules that are detrimental in terms of crop yields and animal growth (Dwyer et al., 2014). Another promising reuse of pomace capitalizes on the recovery of primary and secondary metabolites, including polysaccharides, phytochemicals, dietary fibers, along with other health-promoting nutrients (Iqbal et al., 2021).

Polyphenols have been associated with interesting antioxidant, vasodilatory, antithrombotic, cardioprotective, anti-inflammatory, anticancer and antimicrobial bioactivities (Kato-Schwartz et al., 2020; Zhu et al., 2019; Peixoto et al., 2018). More recently, grape pomace polyphenols and their polysaccharide conjugates have been proven to exert antidiabetic effects (Campos et al., 2021).

Thus, not surprisingly, the development of efficient and, at the same time, environmentally friendly, extraction methods for polyphenols from pomace has mainly attracted the interest of scientists. Among such techniques, microwaves, ultrasound, high hydrostatic pressure, pulsed electric fields, enzymes, and deep eutectic solvents seem to be quite

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promising (Calderón-Oliver & Ponce-Alquicira, 2021). The combination of the above techniques with other strategies, including micro- and nanoencapsulation, turned out to stabilize beneficial compounds (Calderón-Oliver & Ponce-Alquicira, 2021). However, even though some of the reported extraction technologies have already been scaled up to an industrial level in the frame of circular economy, their industrial use is still far from being imminent.

Although anthocyanins and their derivatives certainly represent the major phenolics recovered from red grape pomace, other polyphenols such as flavanols, flavonols, condensed tannins, stilbenes and lignans have been detected in pomaces and reused in pharmaceutical and food industries (García-Lomillo & González-SanJosé, 2017). In this context, it is well documented that polyphenol-rich plant extracts have been employed as safe and clean alternatives to synthetic additives in order to delay food spoilage, retard microbial growth as well as fat deterioration, thus ultimately extending the shelf-life of products (Pateiro et al., 2021). Moreover, polyphenols are successfully used even in the cosmetic segment, as it is well-documented for phenols recovered from olive mill wastewater that are employed as UV boosters (Galanakis et al., 2018).

However, it is now clear that the impressive potential of health-related beneficial effects shown by grape pomace extracts, spanning from anti-inflammatory to antidiabetic (Campos et al., 2021) and antiviral activities (Huang et al., 2016), cannot be ascribed exclusively to the presence of polyphenols.

The secondary metabolism of a single plant is rarely focused on a single class of compounds and, just to cite a well-known example, the multitude of bioactivities associated to *Lamiaceae* plants (e.g. rosemary, sage, thyme, lemon balm or wild marjoram) (Sharma et al., 2020) is due to phenolic compounds, such as rosmarinic acid, but to a great extent also to triterpenoids such as oleanolic and ursolic acids (Lesellier et al., 2021). Accordingly, triterpenoids are emerging as important dietary supplements and they are experiencing a remarkable market growth, especially in the nutraceutical sector (Farzan et al., 2023). Oleanolic acid is a pentacyclic triterpenoid with a wide distribution in the plant kingdom, and it is a major component of the grape berry cuticle (Castellano et al., 2022) with antitumor, antibiotic and even antiviral properties (Lin et al., 2016). As demonstrated in some studies conducted during the COVID-19 pandemic, the molecule turned out to interfere with the interaction of the spike protein receptor binding domain with the carboxypeptidase domain of the Angiotensin Converting Enzyme (ACE2) (Carino et al., 2020). Furthermore, oleanolic acid can improve the structural integrity of mitochondria and endoplasmic reticulum in liver and pancreas of animals fed with HFF diet (high-fat diet plus fructose) (Wang et al., 2018) and showed both antioxidant and anti-inflammatory effects, reducing cartilage degeneration caused by hyperglycemia condition (Li et al., 2021). Equally interesting is the capacity of this triterpenoid of targeting many end points of anti-diabetic relevance by improving the insulin response, by preserving the functionality of pancreatic beta-cells and by generally protecting from diabetic complications (Castellano et al., 2022). Thus, oleanolic acid seems to be a promising natural small molecule which could be used as supplement in diets for patients affected by diabetic and metabolic syndromes.

It is interesting to emphasize that oleanolic acid has been recently identified in Aglianico (*Vitis vinifera* L.) red wine but in concentrations well below those detected in grape samples of the same cultivar (Forino et al., 2019). Aglianico grapevine is largely grown across Southern Italy for the production of renowned red wines awarded the national highest wine classification, DOCG designation (Denominazione di Origine Controllata e Garantita); nonetheless, just a few reports are currently available on the chemical characterization of Aglianico grape varieties (Forino et al., 2019; Arapitsas et al., 2020) as well as on the bioactivities of the derived pomaces. In this regard, very recently some studies describing the antimicrobial, anti-proliferative and pro-apoptotic effects of Aglianico grape pomace extracts have been published (Caponio et al., 2022), but no information on the metabolites responsible for this activity was provided.

Thus, with the purpose of contributing to fill in the above-mentioned knowledge gap, we decided to conduct a full chemical characterization of the bioactive secondary metabolites of the grape pomace deriving from the vinification process of Aglianico with a specific focus on oleanolic acid. In fact, on account of its reduced solubility in hydroalcoholic solutions, such as wine, it was reasonable to assume that large amounts of oleanolic acid could be accumulated in red grape pomaces. The adopted analytical strategy was mainly based on a combined untargeted and targeted approach by means of NMR and Mass Spectrometry (MS). In particular, the untargeted analysis was aimed at identifying possible major bioactive compounds that would turn out to be worthy to recover and reutilize in the dietary, pharmaceutical and nutraceutical sectors, as described above (Annunziata et al., 2020; Badolati et al., 2020; Cecchi et al, 2019).

2. Materials and methods

2.1. Chemicals and reagents

All solvents used in this study were of HPLC grade or higher. Malvidin-3-*O*-glucoside, (+)-catechin, (-)-epicatechin, caffeic acid, quercetin, *trans*-resveratrol, gallic acid, formic acid, acetonitrile, ethanol (EtOH), methanol (MeOH), *n*-hexane, dichloromethane, butanol, ethyl acetate (EtOAc), boric acid, amino acids standards, sodium azide, and diethylethoxymethylenemalonate (DEEMM) were purchased from Sigma-Aldrich (Milan, Italy). Aqueous solutions were prepared with Milli-Q water from Millipore (Bedford, MA, USA).

2.2. Grape pomace collection, extraction and chromatographic separation

Aglianico (*Vitis vinifera* L. cv) grape pomace was obtained from the Taurasi area - Avellino, Italy in late November of 2021. Fresh pomace (2000 g), constituted by skins, seeds, stalks and residual pulp, was collected after the pressing stage to obtain a homogenous sample. 1480 g of fresh pomace were immediately lyophilized, thus affording 418 g of dry biological material. From this quantity of dry pomace, 99.48 g were subjected to extraction as described below. The lyophilized material was grounded by a laboratory blender and extracted twice at room temperature (to avoid degradation of the extracted compounds) with 1.0 L of H_2O:EtOH 2:8 (v/v) overnight. The use of acidified solvents was avoided, since in literature it is reported that either mineral or organic acids should not be used in order to reduce the risk of degrading anthocyanin derivatives or producing anthocyanidins from flavanols and proanthocyanidins (Revilla et al., 1998). After extractions, the dry weight was 91.54 g. The hydroethanolic extract was concentrated to remove residual water and partitioned twice in the order against *n*-hexane 200 mL, EtOAc 300 mL, and *n*-butanol 300 mL. This order of partitioning solvents allowed the extraction of compounds with increasing polarity. Typically, hexane extracts fatty acids (if present) and apolar terpenoids, EtOAc extracts compounds with medium polarity and butanol extracts polar organic compounds. More specifically, three different extracts termed fractions A (*n*-hexane phase), fraction B (EtOAc phase) and fraction C (*n*-butanol phase), respectively, were obtained. All of the obtained phases were fully evaporated under reduced pressure at 30 °C using a rotary evaporator. The final dry weight of the *n*-hexane, EtOAc and n-butanol extracts were 0.296 g, 4.95 g, and 2.496 g, respectively.

The EtOAc extract was further separated through a 80-g silica Combiflash column (3.0 mL/min flow) connected to a Teledyne Isco CombiFlash Rf flash chromatography system, eluted with the following gradient elution: dichloromethane:EtOAc 1:1 (200 mL; fraction 1), 100% EtOAc (200 mL; fraction 2), EtOAc:MeOH 9:1 (200 mL; fraction 3), EtOAc:MeOH 7:3 (400 mL; fraction 4).

2.3. LC-MS/MS analyses

LC-HR MS experiments were performed on a hybrid linear ion trap LTQ-Orbitrap XL™ Fourier transform mass spectrometer equipped with an ESI ION MAX™ source (Thermo Fisher, San José, USA) coupled to an Agilent 1100 LC binary system (Palo Alto, USA). Chromatographic separations were achieved using a Kinetex Polar C18 column (100 × 3.0 mm, 100 Å, 2.6 μm). The injection volume was 0.5 mL/min and the mobile phase consisted of a combination of A (0.1% formic acid in water, v/v) and B (MeOH). For fraction 2, the chromatographic method lasted 25 min and the gradient design was as follows: 50% B (3 min), from 3 to 15 min B reached 95%, and hold at 95% for 5 min. For fractions 3 and 4, the chromatographic method lasted 25 min and the gradient design was as follows: an initial 3 min at 10% B, then from 3 to 20 min B reached 95%, which was hold for 5 min. The analysis was carried out in positive ion mode (ESI +). Source conditions were: spray voltage: 3.5 kV (positive mode) and 2.9 kV (negative mode); capillary voltage: 25 V; source temperature: 320 ∘C; normalized collision energy: 25. The acquisition range was *m/z* 150–1500.

Quantitation of analytes was carried out by analyzing extracted ion chromatograms (XICs) relative to each compound to be quantified with 5 ppm mass tolerance. As standards, (+)-catechin, (-)-epicatechin, caffeic acid, quercetin, *trans*-resveratrol, gallic acid were used. For each of these standards, a calibration curve was plotted on the basis of peak areas (triplicate injections) obtained by using six different concentrations (0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 mg/L). The following calibration curve equations were obtained:

(+)-catechin: y = 2.36e-6x-0.0032 (R^2 = 0.99; LOD = 0.025 μg/μL; LOQ = 0.0850 μg/μL);

(-)-epicatechin: y = 2.02e-6x-0.0004 (R^2 = 0.99; LOD = 0.022 μg/μL; LOQ = 0.0076 μg/μL);

caffeic acid: y = 0.00344x-0.1970 (R^2 = 0.99; LOD = 0.0007 μg/μL; LOQ = 0.0025 μg/μL);

quercetin: y = 9.41e-5x-0.0076 (R^2 = 0.99; LOD = 0.0023 μg/μL; LOQ = 0.0076 μg/μL);

trans-resveratrol: y = 3.73e-5x + 0.0026 (R^2 = 0.99; LOD = 0.0199 μg/μL; LOQ = 0.0663 μg/μL);

gallic acid: y = 0.0015x-0.0087 (R^2 = 0.99; LOD = 0.0012 μg/μL; LOQ = 0.0054 μg/μL).

The remaining analytes for which a standard was not used, on the basis of chemical similarity, it was assumed they shared the same molar response as the standard representative of the chemical class. Specifically, (+)-catechin was used for quantifying catechin-3-*O*-gallate and procyanidin dimers and trimers; (-)-epicatechin was used for quantifying epicatechin-3-*O*-gallate; quercetin was used for quantifying myricetin; caffeic acid was used for quantifying caffeoyltartaric acid; gallic acid was used for quantifying syringic acid; and *trans*-resveratrol was used for quantifying resveratrol dimer and trimer.

2.4. NMR analysis

^{1}H (600 and 700 MHz) NMR spectra were measured on a Bruker spectrometer by using a Norell Select Series 5 mm NMR tubes. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0; CD_3OD: δ_H 3.31, δ_C 49.3 ppm). To quantitation purposes 5 μL of pyridine as a standard were added to the NMR tube containing the compound to quantitate. ^{1}H NMR spectrum of the obtained solution was acquired with a d1 value of 7.0 sec. Representative NMR signals were chosen on the basis of resolution and their area was measured by integration, divided by the number of the protons generating them, and finally converted into the relative number of moles by comparing the obtained area with that of the pyridine protons, for which the number of moles was known.

2.5. High-Performance liquid chromatography analyses of anthocyanins

The separation of the monomeric anthocyanins, contained in the initial hydroethanolic extract of grape pomace, was performed according to the OIV method of analysis (OIV, 2020) by using an HPLC Agilent 1260 infinity II LC apparatus (Santa Clara, USA) consisting of a binary pump, two-channel degasser unit, a diode array detector-DAD (G7114A), and a manual injector equipped with a 20-μL loop. A column heating device set at 40 °C, with a C18 column, Synergi 4 μm Fusion – RP 80 (250 × 3.0 mm, 4 μm particles diameter) with a pre-column was used. All the samples were filtered through 0.45-μm filters (Durapore membrane filters, Millipore-Ireland) into glass vials and immediately injected into the HPLC system. Detection was performed by monitoring the absorbance signals at 518 nm. Data collection and analyses were performed through the software OpenLAB CDS ChemStation Edition (Agilent Technologies, Santa Clara, USA). Elution was carried out by using a flow rate of 0.80 mL/min. Eluents were as follows: A consisted of water milli-Q (Sigma Aldrich)/formic acid (Sigma Aldrich ≥ 95%)/ acetonitrile (Sigma Aldrich ≥ 99.9%) (96:1:3) v/v/v, and B consisted of water/formic acid/acetonitrile (49:1:50) v/v/v. The following gradient was used: initial conditions 94% A and 6% B, after 15 min the pumps were adjusted to 70% A and 30% B, at 30 min to 50% A and 50% B, at 35 min to 40% A and 60% B, at 41 min through the end of analysis, to 94% A and 6% B (Forino et al., 2022). For calibration, the external standard method was used: the calibration curve was plotted for the malvidin-3-*O*-glucoside on the basis of peak area. Concentrations were expressed as mg/L of malvidin-3-*O*-glucoside. All experiments were carried out in duplicate and two analytical replicas were performed. The following calibration curve equation was obtained: y = 5E-06x; R2 = 0.999; LOQ = 2 mg/L; LOD = 0.6 mg/L.

2.6. High-Performance liquid chromatography analyses of amino acids

Amino acids were determined by the methods described by Gómez-Alonso et al. (2007) and by Ortega-Heras et al. (2014) with some modifications as reported below. 10-gram dry pomace was extracted with 130.0 mL of a mixture of acetonitrile/MeOH/ultra-pure water (35:35:30, v/v/v) under a 30-min stirring. The obtained extract was filtered through filter paper and derivatized according to the reported method. In more detail, aminoenone derivatives of amino acids were obtained by reaction of 1.75 mL of a borate 1 M buffer solution, 750 μL of MeOH, 1 mL of the grape pomace extract, and 30 μL of DEEMM in a screw-cap test tube. The mixture was homogenized and placed in an ultrasound bath for 30 min. The sample was then heated at 70 °C for 2 h to eliminate the excess DEEMM and other reagent products. Before the injection, the samples were filtered through 0.45 μm filters (Durapore membrane filters, Millipore-Ireland) into a glass vial and injected into the HPLC system. Analyses were performed with the HPLC apparatus previously described. The chromatographic separation was carried out on a reverse-phase Agilent Infinity Lab Poroshell 120 EC-C18 column (3.0 × 150 mm- 2.7 μm). The solvents were (A) 25 mM acetate buffer (pH 5.8) with 0.02% sodium azide and (B) acetonitrile/methanol (80:20). The gradient used was: 100% solvent A, after 20 min the pumps were adjusted to 95% A and 5% B, at 30.5 min 90% A and 5 % B, at 33.5 min 83% A and 17% B, at 65 min 60% A and 40% B, at 73 min 28% A and 72% B, at 78 min 18% A and 82% B, at 80 min until the end of analysis 100% B. The flow rate was 0.5 mL/min. After a 10 min equilibrium period, the next sample was injected. For calibration, the external standard method was used: the calibration curve was plotted for L-2-aminoadipic acid on the basis of peak area (Gómez-Alonso et al., 2007). The following calibration curve equation was obtained: y = 0.0045x + 0.7612 (R^2 = 0.99; LOD = 0.00013 μg/μL; LOQ = 0.00039 μg/μL). Concentrations were expressed as mg/L. All experiments were carried out in duplicate and two analytical replicas were performed, in order to assess the variability of results.

2.7. Measurement of mitochondrial activity

Mitochondrial activity of the EtOAc extract and of the fractions deriving from its purification was measured in C2C12 cells using the MitoTracker® Red CMXRos probe (Thermo Fisher Scientific), as already described (Riccio et al., 2018). Briefly, the probe was diluted in DMEM to a final dye working solution of 100 nM. C2C12 were washed twice in PBS before being incubated with the probe for 60 min in a cell incubator set to 37 °C and 5% CO2. Upon incubation, cells were washed three times in DMEM and once in PBS to be then fixed in 3.7% formaldehyde for 30 min. MitoTracker fluorescence was measured using a Perkin Elmer Envision 2105 Multiplate Reader (Perkin Elmer) with the following parameters: λ excitation 579 nm, λ emission 599 nm. Cells were then permeabilized in 0.1% Triton X-100 in PBS, and stained with the nuclear dye DAPI. DAPI fluorescence was measured with λ excitation 351 nm, and emission 450 nm. MitoTracker fluorescence was normalized with DAPI fluorescence in order to correlate mitochondrial activity to total number of cells.

2.8. Measurement of glucose uptake

The glucose uptake activity was evaluated for the EtOAc extract and for the fractions deriving from its purification. C2C12 cells were seeded (5 10^3/well) on a 96-well black bottom microtiter plate with (Perkin Elmer, Waltham, USA) in a final volume of 100 μL/well of growth media. When cells achieved 80–90% of confluency, the culture media was carefully removed and replaced with 100 μL of HBSS containing 100 μM, 2-Deoxy-Glucose (2-DG), 0.4 g/L BSA, and 1.27 mM CaCl2 (without any growth factors or FBS) and when indicated the compounds to be tested, as already described (Lapi et al., 2020). Cells were incubated for one hour at 37 °C, the reported temperature to achieve the better vitality of cells. The same HBSS was then further supplemented with 64 μM the fluorescent probe 2-NBDG. After 45 min of incubation, plates were washed twice in PBS. Uptake of 2-NDBG was determined using Perkin Elmer Envision 2105 Multiplate Reader (Perkin Elmer) with the following parameters: λ excitation at 471 nm, λ emission at 529 nm, and monochromator cut off at 360 nm. Following the determination of 2-NDBG, cells were fixed in 3.7% paraformaldehyde for 30 min, permeabilized in 0.1% Triton X-100 in PBS, and stained with the nuclear dye DAPI (100 μM). DAPI fluorescence was measured with the following parameters: λ excitation 351 nm and λ emission 450 nm. Normalized refers to the ratio between intracellular 2-NDBG fluorescence and DAPI fluorescence.

2.9. Statistical analysis

For pharmacological studies, comparisons and differences were analyzed for statistical significance by one-way ANOVA and Bonferroni multiple comparisons test with a single pooled variance. Statistical analysis was performed using GraphPad Prism (GraphPad Software 7.03, San Diego, USA). Values were considered statistically different when p value were * < 0.01, *** < 0.001, **** < 0.0001; and considered not statistically (ns) different when p value was > 0.05.

3. Results and discussion

3.1. Chemical analysis of grape pomace

Grape pomace samples from Aglianico (*Vitis vinifera* L. cv) were collected in the autumn of 2021 from the Taurasi area (Avellino, Italy), renowned for the production of a high-quality red wine with the Controlled and Guaranteed Designation of Origin (Taurasi DOCG). Pomace was lyophilized soon after collection to minimize the degradation of organic molecules. An aliquot of the lyophilized grape pomace was subjected to different and subsequential extractions with solvents with increasing degree of polarity, thus obtaining three different fractions, named A, B and C, as detailed in Materials and methods. These fractions were all subjected to preliminary NMR-based analyses, a crucial step to identify potential phytochemicals worth to be recovered in the frame of circular economy. ^{1}H NMR spectrum of A contained typical chemical shifts of unsaturated fatty acids, and because of their estimated small recoverable amounts the fraction was no further analyzed. Fraction C turned out to be constituted mainly by glycerol, carbohydrates (signals attributable to glucose and fructose were detected), organic acids (tartaric, malic and citric acids) and amino acids, whose quali-quantitative content was determined by the HPLC-based method as reported below.

Particularly interesting was fraction B. In its ^{1}H NMR spectrum diagnostic signals of triterpenoids, flavan-3-ols, anthocyanins, and polymeric pigments were detected. To support this preliminary assignment and identify such metabolites, fraction B was subjected to chromatographic separation through an 80 g-Silica Combiflash column, from which four different fractions were collected and analyzed by NMR spectroscopy. Once again, all of the four obtained fractions were analyzed by NMR spectroscopy. Fraction 1 turned out to be composed by a pure triterpenoid compound, whose chemical identification was conducted by extensive mono- and bidimensional-700 MHz NMR analysis (^{13}C NMR, COSY, TOCSY, ROESY, HSQC and HMBC) without further purification (Fig. 1). The comparison of the obtained NMR data with those previously reported (Forino et al., 2019) led us to unambiguously characterize the isolated triterpenoid as oleanolic acid. Our structural hypothesis was also supported by High Resolution ESIMS ($[M + H]^+$) analysis that afforded an ion peak at *m/z* 457.3664 corresponding to $C_{30}H_{49}O_3$ (calculated for $C_{30}H_{49}O_3$ 457.3676). NMR spectroscopy also allowed an accurate quantitation of oleanolic acid, by using pyridine as an internal standard, a solvent selected because its proton resonances did not interfere with those of oleanolic acid. Thus, fraction 1 turned out to contain 160.1 ± 3.7 mg of oleanolic acid, a relevant piece of information to evaluate the potential recoverable amount of this metabolite. Hence, oleanolic acid accounted for about 3.2% of the dry weight of the EtOAc extract (4.96 g) and about 2% of the dry weight of the initial hydroethanolic extract (7.94 g) deriving from 100 g of dry grape pomace. The concentration of oleanolic acid in fresh Aglianico pomaces can be estimated to hover around 0.45 mg/g, a value consistent with a reported estimation of 0.85 mg/g of oleanolic acid in Aglianico berry skins (Forino et al., 2019). Our NMR-based investigation did not highlight any other triterpenoids apart from oleanolic acid, at least at concentrations detectable by NMR. Hence, a targeted analysis was conducted by means of LC-MS in positive ion mode. It allowed us to detect just a single ion peak at 14.52 min at *m/z* 457.3664 ($[M + H]^+$) attributable to oleanolic acid. This observation was instrumental in ruling out the occurrence of further oleanolic acid isomers, such as ursolic or betulinic acid. At trace levels, we detected an ion peak at *m/z* 471.3817 ($[M + H]^+$) corresponding to $C_{31}H_{51}O_3$ (calculated for $C_{31}H_{51}O_3$ 471.3833) that was tentatively assigned to oleanolic acid methyl ester on the basis of previous reports (Pensec et al., 2014). Other triterpenoid derivatives, including aldehyde-containing analogues, detected in some grape cultivars (Pensec et al., 2014), were unsuccessfully searched for.

In regards to fractions 2 and 3, preliminary NMR-based investigations brought to light the presence of typical resonances of flavan-3-ols, including (+)-catechin, (-)-epicatechin moieties in both fractions 2 and 3, and NMR signals diagnostic of anthocyanin units in fraction 3. To the aim of better defining the polyphenol content of either fraction, we resorted to LC-MS analysis, since it mainly served the purpose of an accurate targeted analysis of the compounds detected by NMR. Considering that the biological matrix under investigation was containing anthocyanin moieties, as also inferred by its red coloration, we resorted to MS experiments performed in positive ion mode suitable for the analysis of all classes of flavonoids. As detailed in Table 1, a number of polyphenols were detected and quantified. In fraction 2, phenolic acids and flavan-3-ol monomers were identified. More

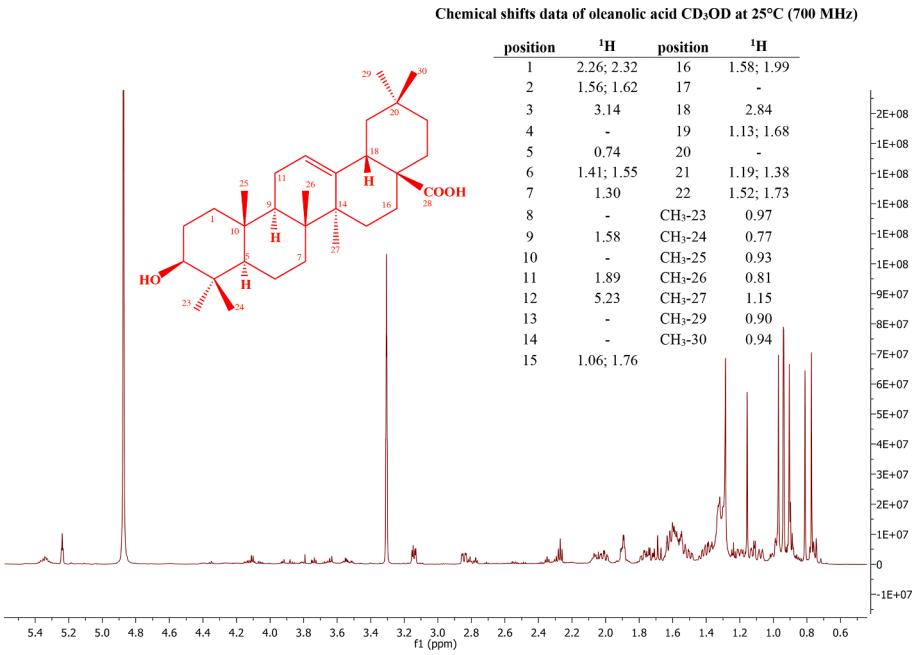

Fig. 1. ^{1}H NMR spectrum (CD_3OD) of fraction 1 containing Oleanolic Acid as major component. In the Table 1H chemical shifts of the molecule are reported.

specifically and consistently with NMR data, the most abundant compounds turned out to be (+)-catechin and (-)-epicatechin, of which only a small fraction occurred as gallate esters. Additionally, among phenolic acids, caffeic acid was the most abundant. Conversely, A-type procyanidins were detected in very small quantities, consistently with previous reports (Unusan, 2020) that have demonstrated that in grapes B-type procyanidins are usually more abundant than their A-type analogs. Indeed, B-type procyanidins were identified in fraction 3, along with flavonols (quercetin and myricetin) and anthocyanins. Among the common anthocyanin monomers occurring in red wines, we could identify and quantify only malvidin-3-*O*-(6-*p*-coumaryl)-glucoside and peonidin-3-*O*-(6-*p*-coumaryl)-glucoside. In order to confirm the monomeric anthocyanin profile of the fraction, an HPLC-based analysis was conducted on the initial hydroethanolic extract of grape pomace according to the OIV method (OIV, 2020) for the analysis of native anthocyanins in grapes and wine. This technique was selected on account of its enhanced selectivity and specificity for anthocyanins. Again, HPLC peaks relative to malvidin-3-*O*-(6-*p*-coumaryl)-glucoside and peonidin-3-*O*-(6-*p*-coumaryl)-glucoside in the extract as major components were observed. HPLC peaks relative to malvidin-3-*O*-glucoside as well as to peonidin-3-*O*-glucoside, which are in the order the two most abundant anthocyanin monomers in the Aglianico red grapes, were detected but below their quantification limit. This datum is of a certain importance to enologists, as it could prompt studies on the extraction kinetics of anthocyanins from grapes into musts. This would suggest the best time to end the maceration process as to possibly avoid the release from crushed grapes of undesirable quantities of molecules, including tannins, that could affect the quality of the finished wines. Finally, by the NMR-based analysis it was ascertained that polymeric pigments had accumulated in fraction 4, as inferred from either the detection of chemical shifts typical of anthocyanin moieties and the drift of the ^{1}H- spectrum baseline. This was not surprising as the most abundant grape and wine polyphenols occur as polymeric compounds commonly ranging from 1000 to 5000 mg/L in red wines, and thus well above the levels of monomeric compounds such as resveratrol, whose concentrations hardly are found above 5 mg/L. This is the reason why the contribution of polymeric polyphenols in terms of health-related effects has necessarily to be taken into account (D'Onofrio et al., 2021). An impressive number of papers has been published on the bioactivities of grape and wine polyphenols either monomeric, oligomeric or polymeric (Li & Sun, 2019). As a consequence, fraction 4 containing essentially polymeric pigments was not further investigated.

3.2. HPLC-DAD analyses of amino acids

The amino acid content and composition, along with polyphenols, is often regarded as a chemotaxonomic tool to differentiate grape varieties. In addition to variety, amino acid composition of grapes is strictly dependent upon pedo-climatic conditions and cultivation practices. We considered a key issue providing insights into the profile of amino acids of the analyzed grape pomace to evaluate their specific residual amounts and assess as to whether it would be advisable a potential recovery. The Aglianico-derived pomace is a fermented one and thus the yeasts play a critical role in determining the amino acidic content. In fact, through the yeast activities, amino acids are converted into secondary metabolites, such as esters, alcohols, aldehydes and ketones that ultimately influence the aroma of wines. The quali-quantitative analysis of amino acids was carried out according to the DEEMM (diethyl ethoxymethylenemalonate)-based method reported by Gomez-Alonso et al. (2007). Table 2 displays the identified amino acids along with their quantitation, conducted as described in Materials and methods. Proline and tyrosine turned out to be the two most abundant amino acids detected in the pomace, in which they occur in comparable and remarkable amounts. In the frame of a possible reuse and valorization of Aglianico grape pomace, it is worth bearing in mind that, over the past decades, tyrosine has been proven to increase dopamine availability, a key metabolite able to enhance cognitive processes (Kühn et al., 2019). This has suggested tyrosine as a food supplement. Also, tyrosine is an interesting compound as a precursor of tyrosol, a powerful bioactive compound (Karković Marković et al., 2019) produced by yeasts during alcoholic fermentation through the Ehrlich pathway. Tyrosol exerts antioxidant activity and it is able to bind human LDL (low-density lipoproteins) (Covas et al., 2003), and thus it can prevent lipid peroxidation and atherosclerotic processes.

In regards to proline, it is not surprising that this amino acid was quite abundant in pomaces, as it cannot be assimilated by yeasts during fermentation. The high content of proline in grape pomace supports its potential as food and feed sources in either human and animal diets.

It is interesting to underline that proline is generally synthesized by plants as a protection against water stress caused by drought and

Table 1
Compounds identified by LC-MS/MS in positive ion mode in fractions 2 and 3 deriving from the chromatographic separation of the EtOAc extract of Aglianico grape pomace.

Compounds	Rt(min)	m/z	Δ(ppm)	m/z fragments	mg/g		
	Fraction 2						
Phenolic Acids							
Gallic acid	3.13	171.0285	−1.753	110.98, 127.01	0.009	±	0.000
Caffeoyltartaric acid	7.88	313.0539	−4.818	150.81, 181.03	0.002	±	0.000
Caffeic acid	12.08	181.0490	−2.791	136.90	0.045	±	0.001
Syringic acid	12.12	199.0596	−2.411	140.05, 155.07	0.021	±	0.001
	Flavan-3-ols						
Catechin	7.78	291.0854	−3.177	127.04, 273.08	0.459	±	0.001
Epicatechin	9.61	291.0851	−4.345	181.05, 273.08	0.526	±	0.008
Catechin-3-*O*-gallate	10.83	443.0958	−3.392	273.08, 291.09	0.086	±	0.001
Epicatechin-3-*O*-gallate	11.34	443.0960	−2.873	273.08, 291.09, 333.14	0.022	±	0.000
Procyanidin dimer A	12.65	577.1319	−3.626	425.09	0.005	±	0.000
Procyanidin dimer A	13.7	577.1320	−3.504	425.09	0.003	±	0.000
	Flavonols						
Myricetin	13.29	319.0439	−2.801	301.03	0.054	±	0.001
Quercetin	14.50	303.0488	−3.726	285.24	0.131	±	0.001
	Stilbens						
Resveratrol dimer	14.32	455.1475	−3.218	349.11, 361.11, 437.14	0.034	±	0.001
Resveratrol trimer	14.79	681.2100	−2.744	587.17	0.010	±	0.000
		Fraction 3					
Flavan-3-ols							
Procyanidin dimer B	6.69	579.1481	−2.819	289.08, 409.09, 453.12, 561.09	0.064	±	0.001
Procyanidin dimer B	6.99	579.1478	−3.268	289.08, 409.09, 453.12, 561.09	0.070	±	0.000
Procyanidin dimer B	7.28	579.1474	−4.045	289.08, 409.09, 453.12, 561.09	0.078	±	0.003
Catechin	7.82	291.0851	−4.035	127.04, 273.08	0.081	±	0.002
Procyanidin dimer B	7.98	579.1476	−3.596	289.08, 409.09, 453.12, 561.09	0.104	±	0.001
Procyanidin dimer B	8.73	579.1477	−3.389	289.08, 409.09, 453.12, 561.09	0.163	±	0.002
Procyanidin trimer B	9.14	867.2118	−1.534	289.07, 579.11	0.011	±	0.000
Procyanidin trimer B	9.49	867.2109	−2.561	289.07, 579.11	0.016	±	0.000
PC2-G isomer 1	9.50	731.1579	−3.722	409.09, 427.10, 579.11, 605.10	0.089	±	0.001
Epicatechin	9.58	291.0856	−2.352	181.05, 273.08	0.154	±	0.001
Procyanidin trimer B	9.68	867.2120	−1.258	289.07, 579.11	0.007	±	0.000
Catechin-3-*O*-gallate	10.83	443.057	−3.483	273.08, 291.09	0.061	±	0.002
Procyanidin dimer B	11.19	579.1478	−3.285	289.08, 409.09, 453.12, 561.09	0.043	±	0.002
Epicatechin-3-*O*-gallate	11.34	443.0960	−2.873	273.08, 291.09, 333.14	0.012	±	0.000
PC2-G isomer 2	11.92	731.1577	−4.050	409.09, 427.10, 579.11, 605.10	0.016	±	0.002
Anthocyanins							
Pn-3-p-coumglc *cis*	13.60	609.1584	−3.131	301.07	0.012	±	0.001
Mv-3-p-coumglc *cis*	13.72	639.1689	−2.944	331.08	0.156	±	0.007
Pn-3-p-coumglc *trans*	13.83	609.1587	−2.491	301.07	0.259	±	0.008
Mv-3-p-coumglc *trans*	13.95	639.1691	−2.757	331.08	0.027	±	0.002

PCn: n units of (epi)catechin; G: galloyl unit. Pn-3-p-coumglc: Peonidin-3-O-(6-O-p-coumaryl)glucoside; Mv-3-p-coumglc: Malvidin-3-O-(6-O-p-coumaryl)glucoside. All the data are expressed as means ± standard deviation, ($p < 0.05$).

salinity. Given that the pomace analyzed in this study was obtained from grapes collected after a quite warm and dry summer, it would be worth to study the possible concentration variation of proline as a function of different summer climatic characteristics.

3.3. *Effect of oleanoic acid enriched fractions on mitochondrial activity of in-vitro cultured C2C12 myoblast*

The isolation of remarkable amounts of oleanolic acid offered us the opportunity to carry out some biological assays to deepen our knowledge on the bioactivity of this interesting triterpenoid. As mentioned above, one the most promising properties of oleanolic acid is its antidiabetic activity. Indeed, obese diabetic mice treated with oleanolic acid showed a decreased fasting blood glucose, improved glucose and insulin tolerance, enhanced insulin signaling and inhibited gluconeogenesis (Wang et al., 2013). Antidiabetic effects of oleanolic acid have been also demonstrated in Type1 Diabetes (T1DB) rats, in which treatments with this molecule significantly reduced the fasting blood glucose levels. (Wang et al., 2011). Accordingly, it has been also shown that oleanolic acid reduces the inflammatory status and dictates a specific macrophage polarization in adipose tissue of obese mice, thus improving insulin resistance, in part through the inhibition of mitochondrial voltage channel VDAC and, in addition, by reducing mitochondrial ROS. Taken together these data suggest that oleanolic acid prevents the activation of NLRP3 inflammasome and MAPK signaling pathway (Li et al., 2021).

In the wake of the above studies and observations, we decided to focus our attention on the mitochondrial and glucose uptake activities of oleanolic acid.

It has been demonstrated that the introduction of lipophilic cations, such as triphenylphosphonium, could target and accumulate the parent compound to the mitochondria of tumor cells enhancing cytotoxicity and selectivity (Zielonka et al., 2017). Recently, triphenylphosphonium cation moiety has been described to improve the selectivity and cytotoxicity of natural pentacyclic triterpenoids by targeting the mitochondria of human cancer cells (Li et al., 2022).

In order to verify the pro-metabolic effect of fractions enriched in oleanoic acid, we investigated their ability to promote mitochondrial activity in C2C12 myoblasts. Mitochondrial activity was measured by using the mitochondrial selective probe MitoTracker CMXRos. The probe accumulates in the mitochondrial intermembrane space and emits fluorescence with an intensity that is correlated to the difference in potential between the mitochondrial matrix and the mitochondrial intermembrane space. This electrochemical potential originates from the activity of the electron transport chain and of the H^+ pumps, providing a measure of mitochondrial oxidative respiration activity. After treatment of C2C12 cells with the EtOAc extract and fractions 1, 2, and 3 at the dosage of 3 μg/mL for 48 h, we observed a statistically significant increase in the mitochondrial activity of C2C12 cells treated

Table 2
Amino acids detected in Aglianico grape pomace. Concentrations are expressed as mg/gr of dry pomace.

	Retention time	(mg/g)		
Glutamic acid	27.02; 26.84	0.010	±	0.003
Serine	31.38		*	
Asparagine	32.35		*	
HO-proline	33.82	0.013	±	0.000
Glutamine	34.47		*	
Histidine	35.01	0.024	±	0.001
Glycine	35.54	0.030	±	0.000
Threonine	36.28	0.006	±	0.001
β-alanine	36.66	0.007	±	0.000
Arginine	37.21	0.010	±	0.001
α-alanine	38.62	0.041	±	0.001
GABA	39.17	0.005	±	0.001
Proline	39.78	0.536	±	0.006
Tyrosine	42.23	0.592	±	0.006
Valine	46.21		*	
Methionine	47.40		*	
Cysteine	50.58; 56.56	0.007	±	0.005
Isoleucine	51.92		*	
Tryptophan	52.07		*	
Leucine	53.04	0.012	±	0.005
Phenylalanine	53.71		*	
Ornithine	58.80	0.008	±	0.004
Lysine	59.73	0.009	±	0.001

All the data are expressed as means ± standard deviation, ($p < 0.05$). * These amino acids were occurring below the estimated quantitation limit.

with fraction 1 and EtOAc extract compared to those treated with vehicles (DMSO 0,1%) (Fig. 2a). We did not observe any significant variations of C2C12 mitochondrial activity upon treatments with fractions 2 and 3 (Fig. 2a). Thus, fraction 1 turned out to be able to induce mitochondrial activity at the dosage of 1.8 μg/mL, with a dose-dependent potency (Fig. 2b). Since, as reported before, fraction 1 almost exclusively contains oleanolic acid, this bioactivity must be ascribed to this triterpenoid. An imbalance between energy production and its utilization may lead to alteration in the cell metabolism, finally resulting in metabolic syndrome; therefore, the demonstrated effect of oleanolic acid on mitochondrial activity may have a relevance in the prevention/treatment of this condition.

3.4. Effect of Oleanoic acid enriched fractions on glucose uptake activity of in-vitro cultured C2C12 myoblast

Once assessed the influence of oleanolic acid on the mitochondrial activities of in-vitro cultured C2C12 myoblast, we moved to test whether crude extract of Aglianico pomace and fractions 1, 2 and 3 were able to promote glucose uptake via membrane glucose transporters (GLUT). Dysmetabolic disorders, which are frequently characterized by insulin resistance and hyperglycemia, have impaired control over the handling of circulating glucose levels. In this context, the GLUT class of membrane transporters plays an essential role to remove glucose from the bloodstream. The majority of these carrier proteins are controlled by insulin and react to insulin stimulation via PI3K signaling. Insulin stimulation can increase the number of GLUT receptors (mostly GLUT4) on the plasma membrane of cells, boost glycolysis, and ultimately enhance the activity of passive transporters (GLUT1) by reducing intracellular glucose levels. Insulin resistance impairs GLUT transporter function, and pharmacological stimulation of these transporters enhances glucose tolerance and reduces hyperglycaemia. The ability of crude extract and fractions to modulate glucose uptake was measured by monitoring the uptake of NBDG, a fluorescent derivative of deoxyglucose covalently bound to the fluorescent chemical nitro blue tetrazolium (NBT). The increase in fluorescence of C2C12 cells incubated with NBDG correlates with the activity of GLUT transporters (Fig. 3). C2C12 cells were treated with 3 μg/mL of the EtOAc extract and fractions 1, 2 and 3 for 48 h. At the end of the incubation, NBDG was added

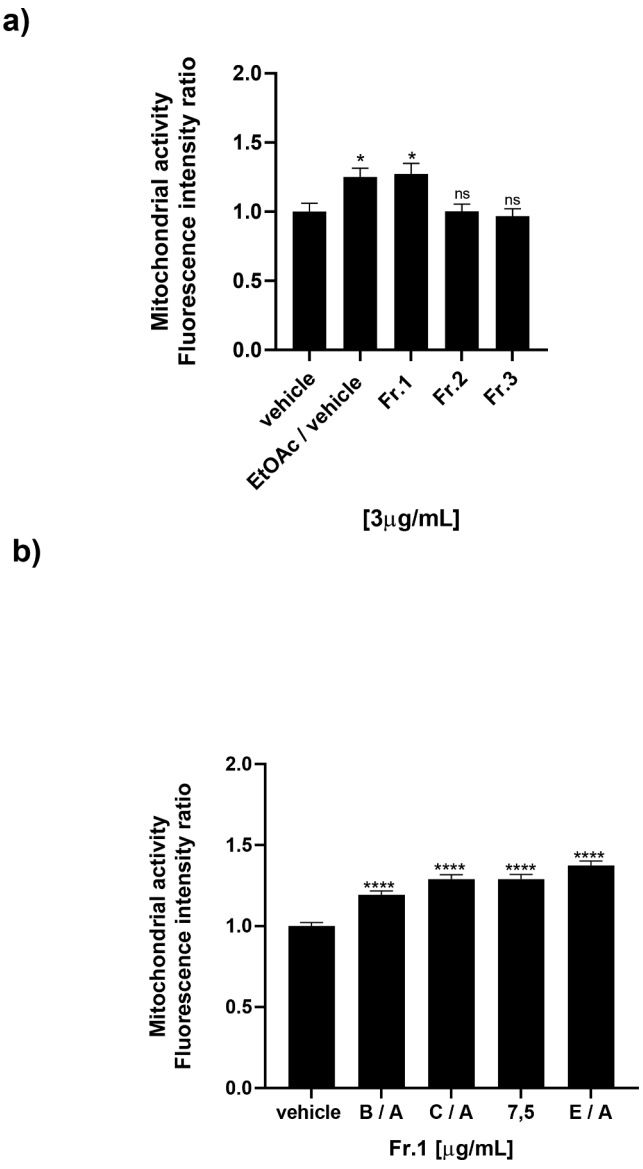

Fig. 2. Oleanoic acid enriched fractions promote mitochondrial metabolism in C2C12 cells. **a)** Mito-tracker Normalized Fluorescence (see methods for details) measured upon incubation of C2C12 cells with crude extract (crude) or the indicated fractions at the concentration of 3 μg/mL for 48 h. **b)** Mito-tracker Normalized Fluorescence measured upon incubation of C2C12 cells with Fr.1 tested at the indicated concentrations for 48 h. Values are expressed as mean ± S.D. of three independent replicates and were considered statistically different when p value were * < 0.01, *** < 0.001, **** < 0.0001; and considered not statistically (ns) different when p value was > 0.05.

to the culture medium. Compared to vehicle (DMSO 0.1%), cells treated with the EtOAc extract and the oleanolic acid rich-fraction 1 showed an increase in NBDG uptake. Thus, modulation of glucose uptake through stimulation of GLUT transporters can be a parallel mechanism that contributes to the beneficial effects of oleanolic acid on diabetes and metabolic syndrome.

4. Conclusions

For the first time, an in-depth chemical analysis of Aglianico grape pomace was conducted by means of different chromatographic (column chromatography, UV–Vis HPLC) spectrometric (HRMS, LC-MS/MS) and spectroscopic (NMR) techniques. This investigation disclosed the presence of a number of bioactive compounds, mainly polyphenols. Among

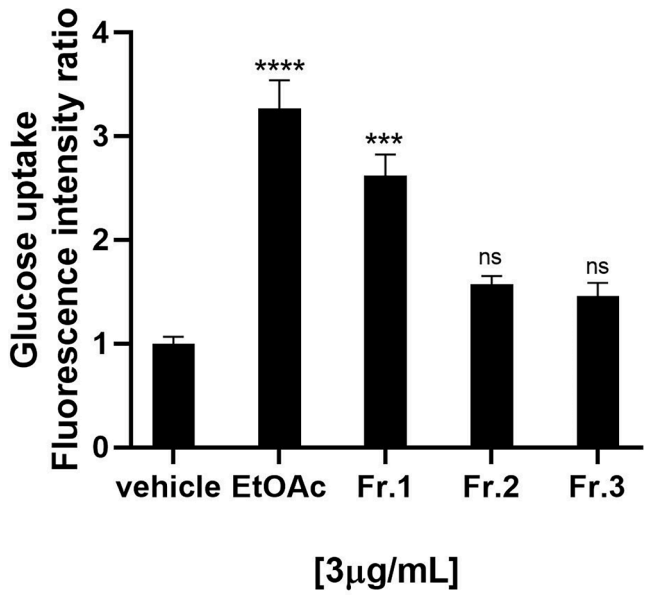

Fig. 3. Oleanoic acid enriched fractions promote glucose uptake in C2C12 cells. 2-NBDG Normalized Fluorescence (see methods for details) measured upon incubation of C2C12 cells with crude extract (crude) or the indicated fractions at the concentration of 3 µg/mL for 48 h. Values are expressed as mean ± S.D. of three independent replicates and were considered statistically different when p value were * < 0.01, *** < 0.001, **** < 0.0001; and considered not statistically (ns) different when p value was > 0.05.

the several anthocyanins commonly occurring in red grapes and wines, only the coumaroyl glucoside derivatives of malvidin and peonidin were found, probably due to their higher hydrophilicity compared to the abundant parent compounds. However, the most relevant outcome of the reported analysis was the isolation in Aglianico pomace of a remarkable amount of oleanolic acid, in the order of 0.45 mg per gram of pomace (fresh weight). This allowed us to verify our initial hypothesis that additional major bioactive metabolites along with typical grape polyphenols are responsible for the reported bioactivities of grape pomace extracts. In fact, oleanolic acid is endowed with a wide selection of biological properties and lately it has been attracting interest for its antidiabetic effects. Our results on the mitochondrial and glucose uptake activities of oleanolic acid on in-vitro cultured C2C12 myoblasts further supported the promotion of this triterpenoids as a promising antidiabetic molecule. More generally, our results clearly demonstrated the utility of an untargeted chemical analysis of food materials and of grape pomace in particular. Indeed, it can bring to light the occurrence of possibly undetected bioactive compounds occurring even in remarkable quantities, such as oleanolic acid that would have been overlooked in by a targeted approach focused on polyphenols.

In perspective, the presented data are expected to prompt further studies aimed at the optimization of extraction and recovery of healthy molecules, and in particular of triterpenoids, from grape pomace by means of sustainable protocols in the frame of circular economy.

CRediT authorship contribution statement

Francesco Errichiello: . **Maria D'Amato:** . **Angelita Gambuti:** Data curation. **Luigi Moio:** Writing – review & editing. **Arianna Pastore:** . **Hekmat AL-Hmadi:** . **Mariano Stornaiuolo:** . **Elena Serino:** . **Orazio Taglialatela-Scafati:** Supervision. **Martino Forino:** Supervision, Writing – original draft.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

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