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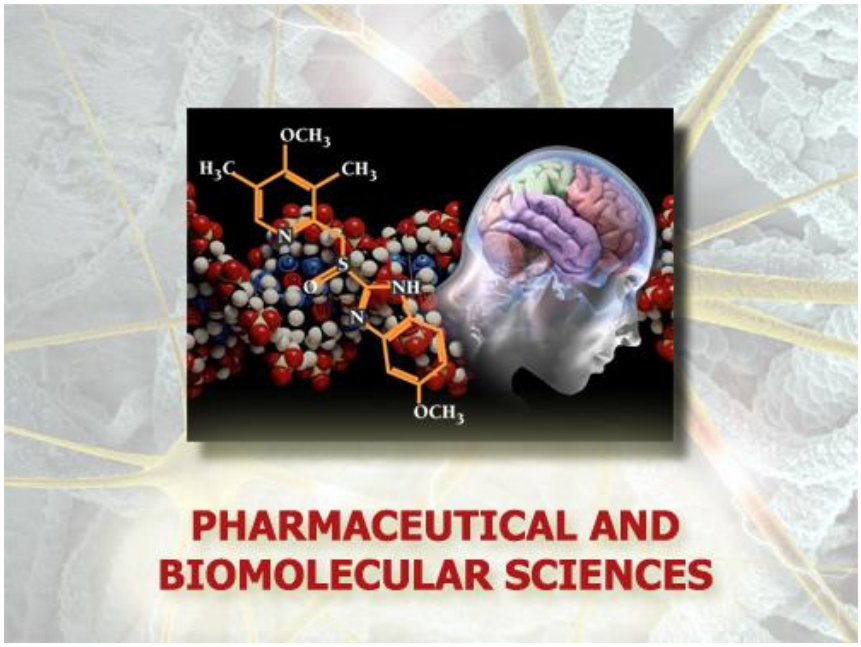
Dottorato in Scienze Farmaceutiche e Biomolecolari (XXXII ciclo)



Isolation and characterization of plant extracts of pharmaceutical, cosmetic and food interest by bioassay guided studies

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XXXII Ciclo

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CONTENTS

GENERAL INTRODUCTION

Our planet is very rich in herbal plant species fundamental for our lives; we cannot survive without the help of plants, and at the same time every herbal product and every plant has some biological effects. Specialized metabolites from plants can be used as defence against herbivores, microbes, viruses or competing plants. They are then important for the plant's survival and reproductive fitness [1]. Moreover they are always present in plants and have many practical applications. Plants are an important source of metabolites used in traditional medicine for their biological properties. Since ancient times, natural products have played an important role all over the world in the treatment and prevention of human diseases. The knowledge of their important healing properties has been passed on over centuries within and among human communities and were successfully used for the discovery of new lead compounds [2,3].

Approximately only 10% of the world's biodiversity has been tested for biological activity, many more useful natural lead compounds are waiting to be discovered. Hence, the importance of having access to this natural chemical diversity [4]. Nowadays, the safety, efficacy and quality of medicinal plants and herbal products is an important topic in developing and industrialized countries. In the last decades, intensive research studies have been carried out to characterize very interesting properties of plant materials to isolate and identify the compounds responsible for those biological activities [5]. In this context, the main goal of this thesis focusing on the wide and complex field of natural products, is the chemical and biological investigation of unexploited parts of food plants with potential health properties: pomegranate (*Punica granatum* L.) and grapevine (*Vitis vinifera* L.).

Chapter 1 of this thesis is based on the literature search related to plants used in traditional medicine, more focused on the uses of pomegranate and grapevine plants, and the state of the art on the study of plants bioactive compounds belonging to their secondary metabolism.

Chapter 2 describes the studies carried out on pomegranate, that is the main matrix evaluated in this doctoral project. Preliminary experiments were carried out on leaves, bark and peel ethanolic extracts which resulted active towards two enzymes involved in HIV-1 replication and were therefore phytochemically characterized. Subsequently, the attention was focused more deeply on the poorly investigated leaves for which the phytochemical pattern was evaluated for plants of different origin, harvesting season and year. To evaluate the constancy of the extracts, phytochemical analyses were performed using HPLC-PDA-ESI-MS/MS and GC-MS systems combined with unsupervised multivariate data analyses. In addition, the phenotypic differences were correlated to the biomolecular analyses with the evaluation of different DNA regions of the leaf samples.

In **Chapter 3,** beside the whole ethanol extract, the attention was also focused on phenolic and triterpenoid enriched fractions, obtained by fractionation with chromatographic techniques. Biological assays were performed on pomegranate leaf extracts, fractions and pure compounds against HIV-1 and also Zika virus whose infection has recently attracted the attention of the medical community since no vaccine or treatments are currently available, [6] while recent studies showed the antiviral potential of polyphenols to inhibit early stages of the infection [7]. At the same time, *in vitro* studies were also carried out on their anticancer activity against acute lymphoblastic leukemia and multiple myeloma cell lines, considering the urgent need of cytotoxic agents that may overcome the ever increasing drug resistance [8,9].

Chapter 4 describes the second investigated matrix, the residues generated by the spring pruning of the grapevine plants (GPRs), an abundant but uninvestigated by-product of the wine supply chain. The study was therefore focused on the polyphenols characterization of GPRs from sixteen red and white *V. vinifera* cultivars from Piedmont (Italy). The results were compared with those obtained for the leaves, these last reported in literature as a good source of bioactive nutraceutical compounds [10]. *In vitro* antioxidant assays were also carried out on the leaves and GPRs, to compare their potential activity. In conclusion, the investigations carried out on the two investigated plants showed that these unexploited herbal parts can be a promising source of active compounds to be used in several fields.

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Chapter 1

Natural products: from traditional medicine to the study of plant specialized metabolites

CHAPTER 1: NATURAL PRODUCTS: FROM TRADITIONAL MEDICINE TO THE STUDY OF PLANT SECONDARY METABOLITES

1.1. Plants used in traditional medicine

Traditional medicine, in according to the World Health Organization (WHO), refers to the knowledge, skills, beliefs and practices based on the theories and experiences of different ancient cultures, with the main and common aim to maintain the human and animal health or, to prevent, diagnose and treat physical and mental illnesses. In contrast with modern medicine based on biochemical and biomolecular theories of illness, traditional medicine includes beside massages and spiritual therapies also medicaments derived from plant, animal and minerals. The 80% of populations living in some countries in Asia and Africa where there is a limited access to modern medicine, uses traditional medicine as the primary source of health care [1]. Human beings considered plants since ancient times; plants and medicinal herbs represent an indicator of ecosystem health [2].

Nowadays, due to the toxicity and side effects of common allopathic medicines and above all the increasing development of microbial resistance to synthetic drugs, the demand of herbal drugs as alternative or complementary solution among population is increasing and the medical community turned to ethnopharmacognosy. Natural products can be economical, in some cases effective and with less adverse effects [3]. Traditional Chinese and Indian medicine are currently the most popular; these countries have been turned into one of the oldest sciences. In the ancient history of humans, the use of medicinal plants was the only choice to treat diseases [4]. As an example, in ancient Persia plants were used as disinfectant, drugs and aromatic agent [5]. More than a tenth of the plant species (over 50.000) find applications in pharmaceutical and cosmetic fields. Their distribution worldwide is not equal and their collection is attributed mainly in developing countries [6] and the rich source of compounds they contain can be used in drug synthesis [7].

Different plant parts can be used, such as fruit, skin, leaf, seeds, root, flowers or even the whole plant, where active compounds are stored. These substances can have direct or indirect physiological effects on living organism therefore they can be used as medicinal agents [8]. Archaeological artefacts indicate that humans cultivated plants as drugs approximately 60.000 years ago [9]. Ancient scripts date back about 5.000 years ago in China, India and Egypt and 2.500 years in Central Asia and Greece where Hippocrates and Aristotle used medicinal plants to treat diseases [10]. In the first century AD, Pedanius Dioscorides, a physician and surgeon, wrote *De Materia Medica*, an encyclopedia with the description of 600 medicinal plants based on scientific studies [11]. One of the main advantages today in the revival interest towards traditional herbal medicine is the possibility to confirm that its long standing use in developing and developed countries for thousands of years as a natural resource can be associated to a sustainable safety. Nevertheless the safety of herbal remedies is nowadays supported by the increasing knowledge of their composition. [6].

1.1.1. Pomegranate in folk medicine

The beneficial properties of pomegranate are mentioned by all main religions and by folk medicine. It has been represented a natural symbol of fertility and fecundity for many cultures [12]. In the ancient Ayurvetic medicine, for thousands of years, the pomegranate has considerably been used as a source of traditional healthy remedies. All parts of the plant were used due to its own qualities and specific healing properties.

The fruit rind and the tree bark of the pomegranate were used as a traditional remedy against dysentery, diarrhoea and intestinal parasites while the juice and seeds derived from the fruit were considered a tonic for the heart, throat, and eyes. In addition they were used for several purposes; such as toning skin, stopping nose and gum bleeds, treating haemorrhoids and firming-up sagging breasts [13].

Pomegranate was widely used as a traditional remedy in the traditional medicine of Sardinian culture. To the decoction of flowers and rind of the pomegranate fruit is attributed intestinal astringency, antidiarrheal and haemostatic properties. Flower preparations were used to block tuberculosis haemoptysis. Some parts of the pomegranate such as fresh root bark and decoction of fruit peel and tree bark were widely used as an effective intestinal vermifuge, in particular against tapewarm in the locations of Nuoro, Oliena, in the land of Bolotana and in the Campidano of Cagliari. Other documented internal uses of fruit peel decoction in the locations of Gairo, Jerzu and Villanosa Strisaili, were as refreshing, anti-inflammatory, purifying, antiodontalgic and eupeptic digestive in the gastric atonia. Bark preparations were used as an antihemorrhoidal, while the enteroclysis of fruit peel decoction, dried fruit part or crushed seeds juice were used as antidiarrheal in Villacidro. Moreover, the fruit peel or bark decoctions were used as an analgesic to treat otitis. Pomegranate leaves were also placed between food patches to treat excessive feet sweating. In the location of Oristano, the elderly women who had lost their teeth, used the decoction of peel in mouth rinsing and also to strengthen the gums [14].

1.1.2. Grapevine in folk medicine

Since ancient times, grapevine has been used in various forms in religion and medicine. In history, the grapevine represented a symbol of life, and wine a symbol of blood, a connection with divinity, which has continued in the Christian religion across millennia. In the early Islamic art, the representation of grapevine meant prosperity, richness, abundance and fertility, while wine indicated blessing and joy [15]. In traditional medicine, the leaves' infusion was used to treat stomach aches, bleeding, hepatitis and diarrhoea, while for external use, the fresh leaves were utilized to heal wound and lance abscesses.

In Palestinian folk medicine, the leaves were used as astringent, tonic, diuretic, antiseptic, blood purifying, antianemia and antihypercholesterolemic agent [16]. In Indian traditional medicine the ripe fruit is considered to be laxative and purgative, fattening, aphrodisiac, diuretic, appetizer and to cure thirst, asthma and blood diseases. The ashes of stem were used to treat pain in joints, swelling of the testicle and piles. The flowers are considered expectorant and useful in bronchitis. The sap of young branches was used as a remedy for skin diseases while juice of unripe fruit was employed in throat affections. The dried fruit, beside food consumption, was also used such as demulcent, cooling, laxative, to relief thirsty, fever, coughs, hoarseness and in wasting diseases.

From the seed was derived a malagma, which represented a folk remedy for condylomata of the joints. In Iran, grape leaves were used in a traditional food and for treatment of diarrhoea and bleeding [17].

1.2. Plants secondary metabolites

Plants are eukaryotic and sessile organisms and, in their evolution, have developed efficient mechanisms to overcome the constrains of living in a "fixed" place. These mechanisms are divided to grow such as light and nutrient intake, to reproduce as pollination and seed dispersal and finally to defend themselves from the surrounding environment [18,19]. Except light collection, correlated exclusively to individual inner morphological and chemical characteristics, all the other factors involve the interaction of plants with their surrounding environment and are represented by abiotic factors, pathogens, heterotrophic or mutualistic organisms and specific interactions with other plants (Figure 1) [19].

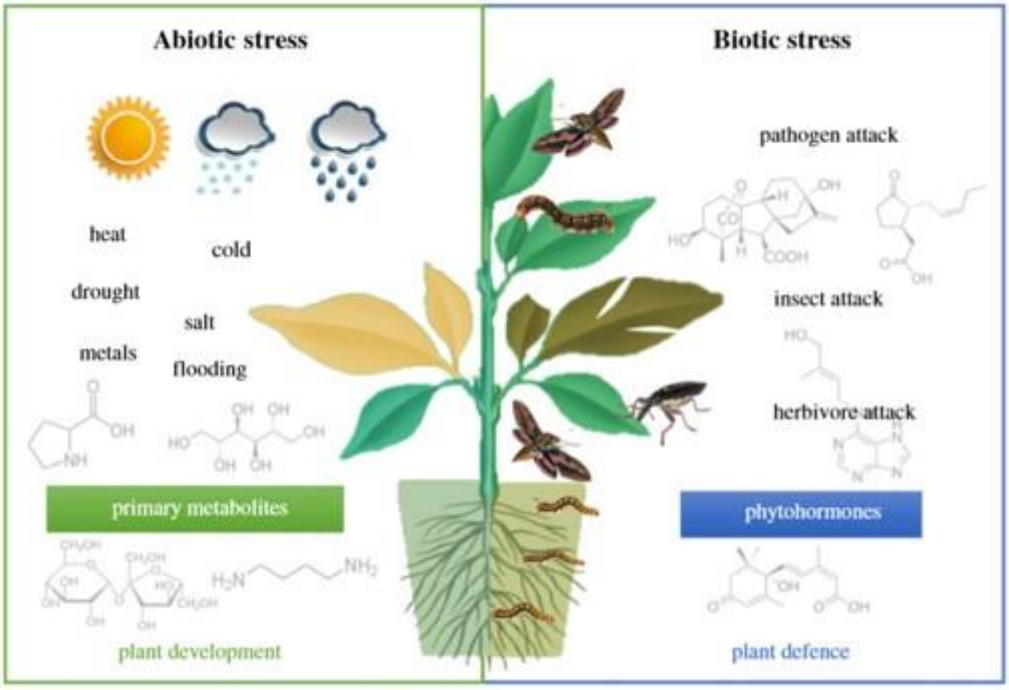


Fig. 1. List of adverse environmental factors (abiotic stress) and plant–attacker interactions (biotic stress) that affect plant growth and development. Plant responses with the production of chemicals from reproductive and vegetative parts in some cases to fight and in others to cooperate with these conditions [20].

The compounds directly related to plant growth and development, fundamental for plant life and involved in essential functions, are called primary metabolites and produced as conservative traits by plants; the compounds not essential for life functions but fundamental to survive in a constantly changing environment, are called secondary (or specialized) metabolites. They are adaptive and variable in the different plant species; in addition, it is proposed that in the early plant evolution stages, the secondary metabolites variability was used for primary functions such as xanthophylls in photoprotection or phytohormones [18].

The main characteristics of primary and secondary plant metabolism are listed in Table 1. Nowadays, scientific studies highlight the great variability in plant secondary metabolism, counting more than 200.000 different chemical compounds expressed as a response to the various environmental conditions [21]. The chemical structures belonging to the different secondary metabolite classes are shown in Figure 2.

Table 1. Differences between primary and secondary metabolism characteristics [18].

Primary metabolism	**Secondary metabolism**
Genes with high stringency controlling essential functions	Genes with high plasticity controlling functions that are under selection pressure of a continuously changing environment
Covers growth and development of the individual	Covers interactions of the individual with its environment
Indispensable	Dispensable for growth and development
	Indispensable for survival in the environment
Universal	Unique
Uniform	Diverse
Conservative	Adaptive

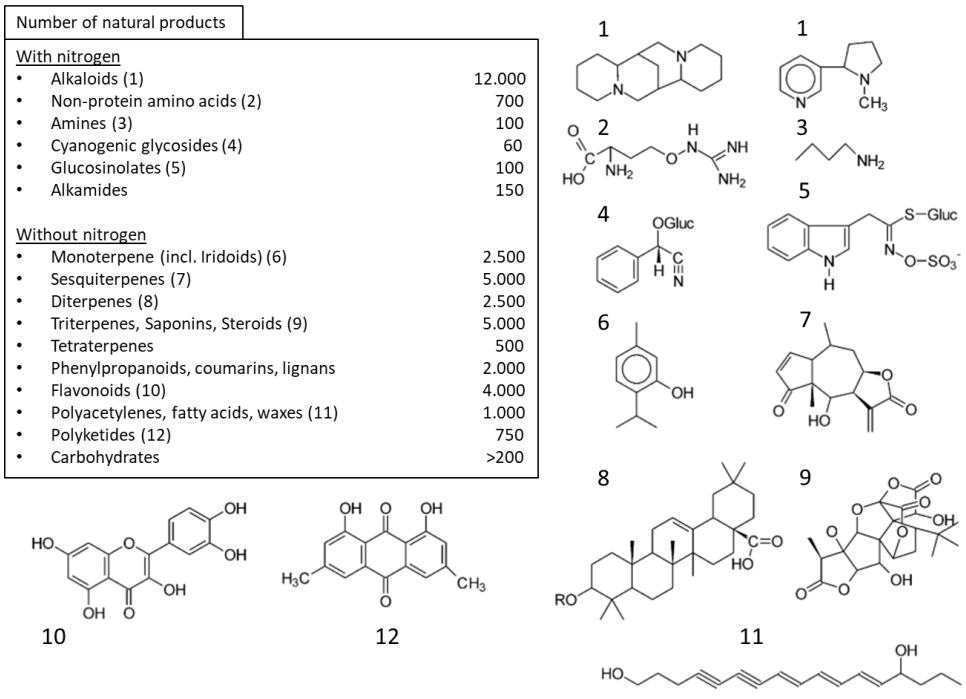


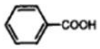
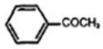
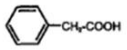
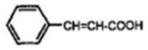
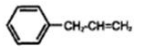
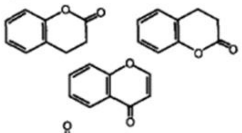
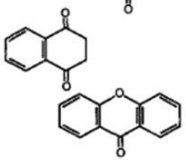
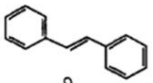
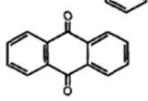
Fig. 2. Example of diverse structures of plant secondary metabolites [22].

Among all secondary metabolites, the phenolic compounds, are highly valuable in human health and nutrition and current literature correlates their dietary intake with a potential decreased risk of cancer and cardiovascular diseases [23]; terpenes are very interesting antimicrobial and anticarcinogenic agents [24,25].

Phenolics are chemical compounds composed by at least one aromatic ring with one or more hydroxyl groups bonded to aromatic or aliphatic structures [26]. These compounds are found predominantly in fruits, herbs, vegetables, leaves, roots and seeds; moreover they play a crucial role in the defence system, reproduction, sensory and structure processing [27]. In higher plants, several thousands of polyphenols have been characterized. Their content is influenced by several conditions such as biotic and abiotic stresses represented by the photoprotection and defence against mechanical damage, pathogens and herbivores. In addition, phenolics are implicate in flower and fruit pigmentation.

They are mainly produced by the shikimate and phenylpropanoid pathways and are composed of one or more aromatic ring backbones with hydroxyl groups or other substituents such as sugars and organic acids [28]. In according to Bravo et al. [29], polyphenols can be classify in at least ten different categories on the basis of their chemical structure, according to Table 2.

Table 2. Representation of the main classis of polyphenolic compounds on the basis of their chemical structure [29].

Class	Basic Skeleton	Basic Structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naftoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans, neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

They can be characterized by simple molecules such as the phenolic acids or complex polymeric structures as the tannins. The latter group of polyphenols can be divided in two classes: condensed tannins called also proanthocyanidins, which are constituted by flavan-3-ols polymers subunits connected via 4-6 and 4-8 interflavan bonds, and hydrolysable tannins, composed by gallic acid esters with a central polyol, generally β-D-glucopyranose. The penta-*O*-galloyl-β-D-glucopyranose is the product of the gallic acid glycosylation reactions and represents the precursor of all hydrolysable

tannins which are again divided in two subclasses: gallotannins derived from galloylation reactions of this precursor derivative and ellagitannins that are formed by 3,4,5,3',4',5'-hexahydroxydiphenoyl (HHDP) moiety derived from oxidation reactions among galloyl residues of the penta-O-galloyl-β-D-glucopyranose molecules [30]. In addition, the ellagitannins hydrolysis releases HHDP from which ellagic acid molecules are forming spontaneously [31].

Figure 3 describes the oxidation of pentagalloylglucose to tellimagrandin II, which represents the primary metabolite in the ellagitannin metabolism and it can subjected to further oxidation reactions; in addition its hydrolysis products are here shown [32].

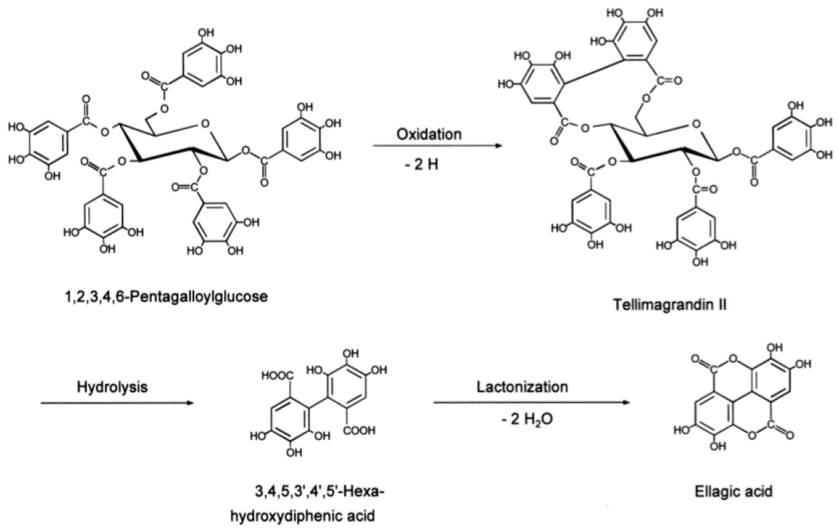


Fig. 3. Formation of the monomeric ellagitannin tellimagrandin II from the oxidation of pentagalloylglucose, and its hydrolysis to hexahydroxydiphenic acid and ellagic acid [32].

The second subclass of hydrolysable tannins are very common in plants representing the widest known group of tannins. Pomegranate and berries such strawberry and raspberry belonging to the Rosaceae family are a rich source of ellagitannins [30]. Currently, several scientific studies attribute to ellagic acid and more generally to tannins, antioxidant, antimicrobial, antiviral and anticancer properties [33].

Phenylalanine and tyrosine, on the action of aromatic amino acid lyases in the shikimate pathway, represent the precursors to the phenylpropanoid pathway, directing carbon efflux from primary to secondary metabolism within the plant [34]. Phenylpropanoids class includes phenolic acids such as caftaric, coumaric and caffeic acid and more complex molecules as lignans, stilbenes and flavonoids. The latter are found in several fruits as pomegranate and grapevine and also in numerous their by-products such as leaves, seeds, bark and peel [35,36]. Figure 4 highlights the different structures originated by the general phenylpropanoid pathway [34].

The structure of flavonoids is the flavan nucleus, formed by 15 carbon atoms arranged in three rings (C6-C3-C6); two of them are aromatic (labelled as A and B ring) and linked by an oxygenated heterocycle, the C ring. The chemical features of ring C define the flavonoid subgroups as reported in Table 3 [29]. Starting the flavonoid pathway, the condensation reaction of coumaroyl CoA and malonyl CoA molecules lead to the formation of a chalcone moiety that spontaneously isomerizes to flavanones. From them, the different modifications on the C ring define the various subclasses of

flavonoids (Table 3). One of these subclasses is represented by dihydroflavonols that can be converted to colourless and unstable pigments called anthocyanidins. Their oxidation and glycosylation originate the anthocyanins, that are coloured and stable molecules and are accumulated in vacuoles giving a blu, red or purple colour to several fruits. Anthocyanins are reducted to flavan-3-ols that can be in a monomeric form such as catechin or in an oligomeric form as condensed tannins. Condensed tannins confer an astringency to fruits hindering their consumption when unripe [37,38].

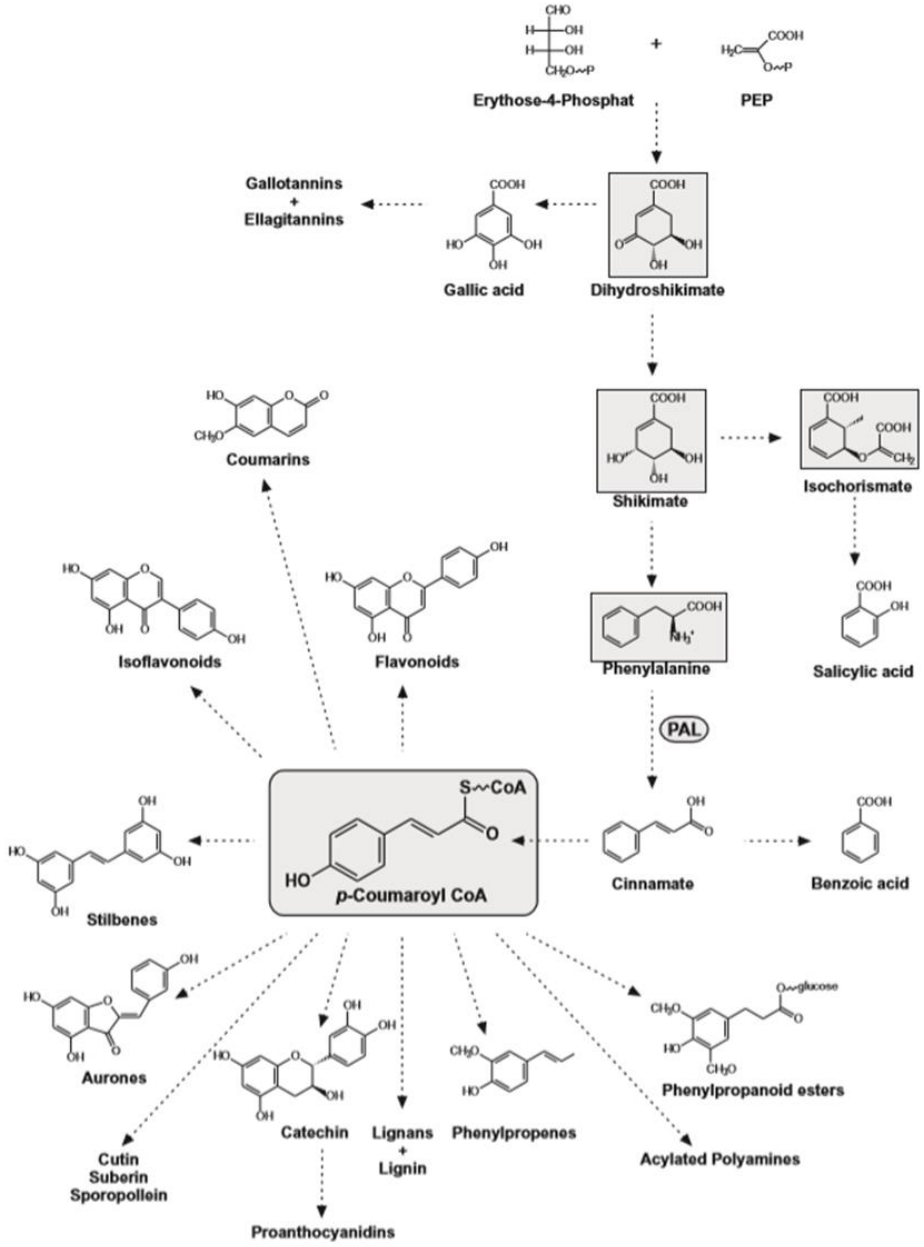
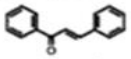
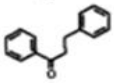
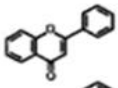
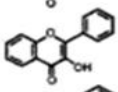
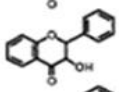
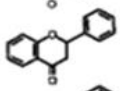
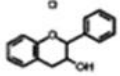
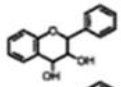
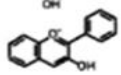
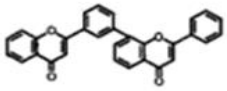
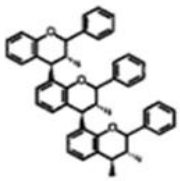


Fig. 4. Diverse structures originated by the phenylpropanoid pathway. Phenylalanine ammonia lyase (PAL) enzyme catalyses the nonoxidative deamination reaction of phenylalanine to trans-cinnamate leading the carbon efflux from the shikimate pathway to the general phenypropanoid metabolism [34].

Table 3. Diverse chemical structures of flavonoid subclasses found in plants [29].

Flavonoid	Basic Structure
Chalcones	
Dihydrochalcones	
Aurones	
Flavones	
Flavonols	
Dihydroflavonol	
Flavanones	
Flavanol	
Flavandiol or leucoanthocyanidin	
Anthocyanidin	
Isoflavonoids	
Biflavonoids	
Proanthocyanidins or condensed tannins	

The terpenoids up to now, represent the largest class of secondary metabolites identified in plants. All terpenoids originates in the cellular cytosol from the mevalonate pathway starting from acetyl CoA, or in the cell plastids starting from pyruvate and glyceraldehyde-3-phosphate [39].
These two pathways have in common the formation of two isomers composed by 5-carbon atoms: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These two molecules are condensed to form the terpene pre-cursors [40].

Terpenoids are therefore classified on the basis of the number of isoprene units (C_5) incorporated and as a consequence by their carbon chain length: molecules with ten carbon atom units are called monoterpenes (C10), sesquiterpenes are characterized by fifteen carbon atom units (C15), diterpenes with twenty (C20), triterpenes with thirty (C30), tetraterpenes with forty (C40), and polyterpenes with more than forty-five (>45), as represented in Figure 5, and in order to increase

the chain length, they are formed through sequential head-to-tail condensation reactions of IPP with DMAPP, geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP) so on and so forth [28] .

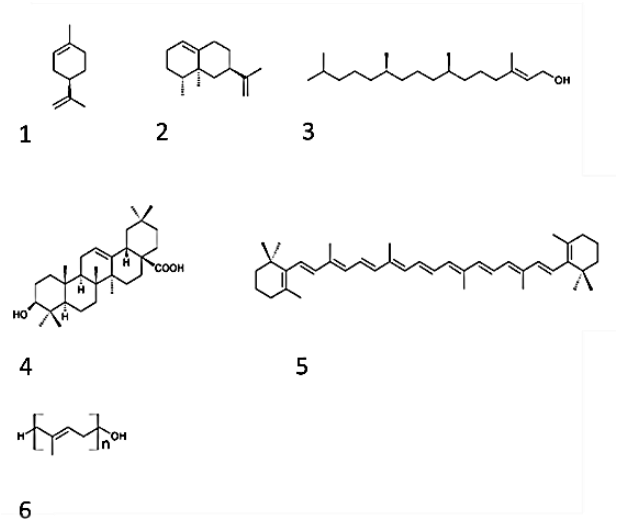


Fig. 5. Diverse chemical structures of terpenoids: 1) *R*-Limonene (C10); 2) Valencene (C15); 3) Phytol (C20); 4) Oleanolic acid (C30); 5) β-carotene (C40); 6) Betulaprenole (C>45) [28].

1.3. Studying the bioactive compounds

These compounds can possess important biological properties as anticancer, antihypertensive, anti-infectives, anti-inflammatory, analgesic, antioxidant, immuno-suppression, for neurological diseases and wound healing treatments [3], and they have a promising future in the improvement of hard-to-treat illnesses, and around the world about half a million plants are present and most of them have not been investigated yet. The huge biodiversity of plants provides inexhaustible chances for new drug discoveries in the standardized plant extracts [7].

One of the main way in drug discovering is represented by bioassay-guided process (Figure 6) where different extracts of plant raw materials are tested and if a biological activity such as antibacterial, antiviral or antiproliferative action is observed, the raw extract is subsequently fractionated to isolate and identify the potential bioactive compounds. Each fractionation step is guided by biological assays. However, not always this process lead to discover and isolate a single bioactive compound because sometimes the activity of the extract is provided by a synergistic effect of its multiple components [6].

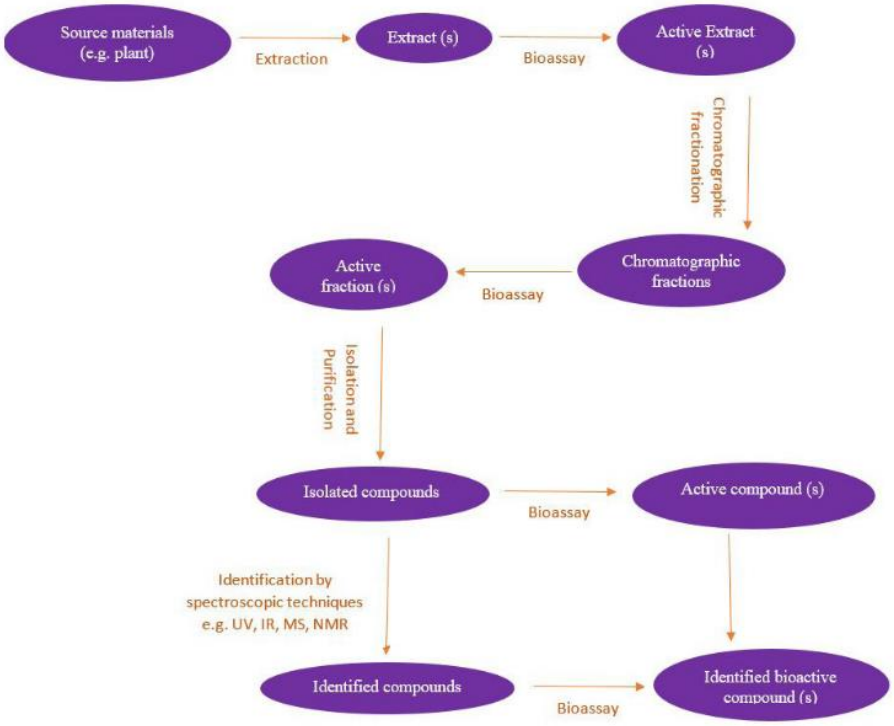


Fig. 6. Traditional process in natural drug discovering: bioassay-guided fractionation [6].

Despite all the benefits that natural products provide to human health, pharmaceutical companies are still reluctant to their large scale use. Some issues and limitations need to be overcome in the future: such as their structural complexity, difficulties and time-consuming of natural product chemistry screening that can cause a large number of artefacts, the event of unknown and unpredictable synergistic actions among different compounds in plant extracts and the poor reproducibility of them due to seasonal changing affecting the plant metabolism. In addition, the uncertain availability of some plant materials and the access to biodiversity due to political problems and the International regulations has to be considered [41].

1.3.1. Phytochemical characterization

The characterization of a plant extract from a phytochemical point of view means to perform a very complex study that involves a multidisciplinary approach. A plant extract is usually a combination of several types of bioactive molecules or compounds with different polarities and their separation and characterization represent a big challenge. In order to obtain pure compounds, a number of different separation (chromatographic) techniques are used: column chromatography, flash chromatography, Thin-Layer Chromatography (TLC and HPTLC) and preparative Liquid Chromatography (prep. LC)[42]. High Performance Liquid chromatography (HPLC) represents a robust, versatile and widely used technique which can separate, and purify individual components in a mixture of compounds [43]. LC coupled to the different detectors, makes possible to identify and quantify the components of a complex mixture. Nowadays, HPLC is the main used technique in analytical and preparative scale for the quality control of plant extracts; its resolving power allows to rapid processing for fingerprinting study of multi component samples [42,44]. Several studies characterized and quantified secondary metabolites in plant extracts such as phenols, steroids, alkaloids and flavonoids using the HPLC technique [45–49].

Reverse-phase chromatography represent over 65% of the total HPLC separations due to its broad application range. [50]. The non-polar column stationary phase is usually composed by hydrocarbons (i.e. C18 functional groups covalently bonded to the silica particles' surface, while the polar mobile phase is generally represented by water or polar solvents). The non-polar molecules have a greater affinity for the stationary phase and pass through the column more slowly than the polar ones. For the identification of any compounds, a detector is needed and because many natural compounds have some chromophore groups responsible for UV absorption [51], such as phenolics identified at wavelengths of around 190-380 nm [52] using UV-VIS and Photodiode Array (PDA) detectors widely used for their high sensitivity [53]. Figure 7 shows a schematic representation of HPLC system.

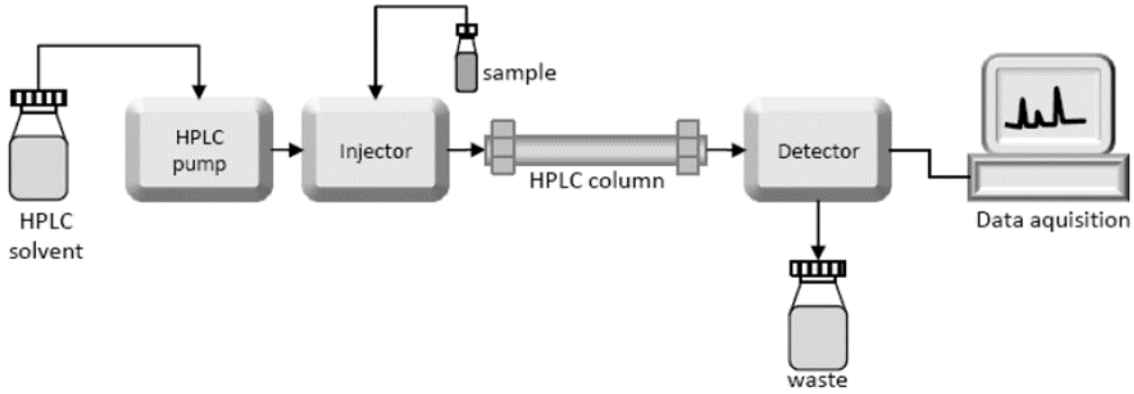


Fig. 7. Schematic representation of the HPLC system [54].

Another important detector coupled with HPLC system is Mass Spectrometer (MS) and the possibility to combine information from a Diode Array Detector (DAD) and Mass Spectrometer Detector (MSD) represents a powerful technique employed for the phytochemical analysis of complex plant extract [55]. Beside the liquid chromatographic separation, these technique combination can facilitate the identification of compounds in plant extracts, in particular when commercial pure standards are not available, providing a partial structural elucidation of the molecules and accurate information about their fragmentation by performing collision-induced dissociation (CID) experiments when tandem Mass Spectrometry (MS/MS) or more (MS^n) sequential stages of MS are applied [56]. Generally, in LC-MS analysis, electrospray ionization (ESI) is the most commonly employed ionization method, which is an eligible source in the phenolic compounds analysis; it is considered a soft-ionization technique that provides little internal energy and high ionization efficiency [56].

Nowadays, several MS instruments for LC-MS-based plant metabolomics are currently available: triple quadrupole (QqQ), quadrupole time of flight (QTOF), quadrupole ion trap (QIT), triple quadrupole-linear ion trap (QTRAP) and quadrupole Orbitrap. Within them, triple quadrupole system (LCQqQ-MS) is eligible in quantitative targeted approach, allowing highly sensitive multiple or selected reaction monitoring (MRM/SRM) analyses and giving a certain absolute quantification of metabolites present in low abundance (Figure 8) [20].

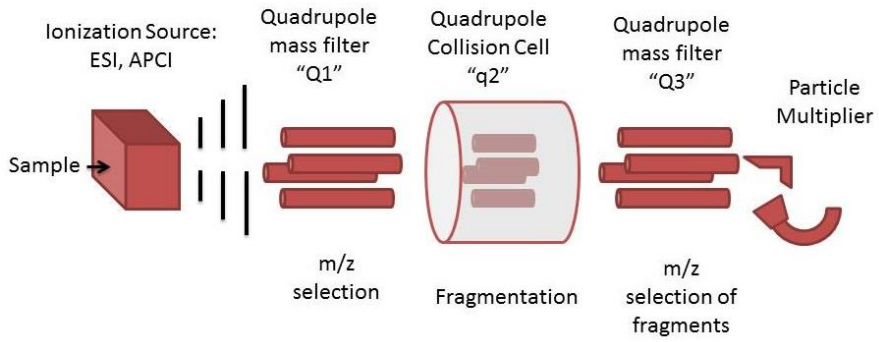


Fig.8. Schematic representation of the triple quadrupole MS/MS system (en.wikipedia.org).

To provide a suitable sample for HPLC analysis, the processing of the raw materials (e.g. dried powdered) and the choice of the extraction solvent (e.g. methanol, chloroform) are very important to the success of the isolation technique [48]. The crude source has to be treated to ensure that the compounds of interest are exhaustively extracted. After extraction, the solid material is usually removed by filtration [42,53] and the filtrate is subsequently concentrated and injected in HPLC for the analysis. When a crude extract analysis is carried out, the use of a guard column is often necessary to protect the lifespan of the analytical column from strongly binding components contained in the extract such as chlorophylls that in long time can compromise its performance [56].

Gas chromatography (GC) combined with flame ionization detector (FID) or MS detector represents another technique in which an inert carrier gas such as helium is used as the mobile phase, widely employed for the analysis of bioactive natural compounds, especially for volatiles compounds such as essential oils and hydrocarbons [57]. GC-MS is used for the structural elucidation of compounds and is characterized by better sensitivity and lower limits of detection and quantification of the analytes [58]. Due to its sensitivity, simplicity and effectiveness in chromatographic separation, is broadly used for qualitative and quantitative analysis of complex mixtures as one of the most important tools in analytical chemistry [59]. A limit that occurs in GC analysis is represented by low volatility molecules, but its use can be extended also to medium-high boiling and medium polarity molecules changing their volatility by proper derivatization reactions such as to their corresponding TMS derivatives using pyridine and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) [60]. Derivatization of the sample can improve the chromatographic separation, the suitability, efficiency and detectability of the compounds [61]. In Figure 9, a schematic representation of a GC-MS system is shown.

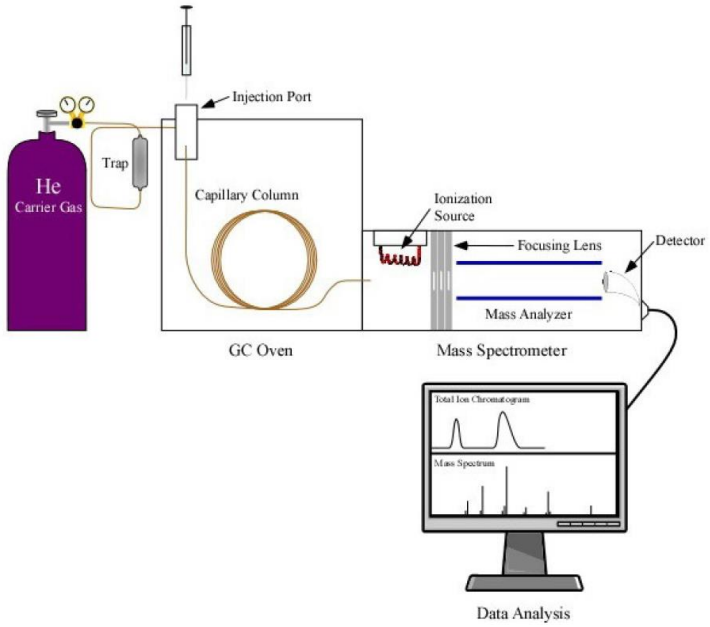


Fig.9. Schematic representation of a GC-MS system [62].

1.4. Biomolecular characterization: DNA barcoding

The identification of different organism on morphological characters often represents a challenging task for taxonomists. In addition, traditional morphological concepts cannot identify biological species which have developed genetic divergences without the presence of morphological differences. In order to overcome this issue, in the last decades, DNA barcoding has been developed as a powerful tool for species discrimination [63]. DNA barcodes are represented by short standard DNA regions known as marker, instead of whole genome. Scientific community and industry are widely using this technique to solve different issues in molecular phylogenetic, taxonomy, biogeography, in the illegal trade control, in food product authenticity and in population genetics [64].

The DNA barcoding is not only used in research of recent biodiversity but also to study the past ecological conditions on Earth. Recently, the use of DNA barcoding has increased in the field of medicinal plants research, it represents a valid technique to authenticate several herbal products in the industrial quality assurance [65,66].

In this field, various degrees of discrepancy are recently reported among expected and actual species identified in the various commercial herbal products. In the light of these studies, the authenticity and quality of herbal products were questioned. The practical limitation of conventional DNA barcoding is restricting this analysis only for the authentication of herbal preparations composed by unprocessed plant materials because after processing steps such as using solvents, DNA can be lost, mixed or degraded. Many factors influence a representative evaluation of a complex mixture, such as the quality and type of raw materials [67]; moreover, false positive results given by any amplifiable DNA and false negative results if the DNA is degraded or lost due to manufacturing or post harvesting treatments, may affect the final DNA analysis [66].

The DNA-based identification procedure is represented by the focus on plant sampling, barcode regions, DNA extraction, purification, amplification and comparison with the sequences reference database [68].

Figure 10 reports the procedure of DNA barcoding and metabarcoding techniques as well as a graphic representation of the potential strengths and weaknesses of source tissue in the development of reference libraries for DNA barcoding. The DNA barcoding combined with high-throughput sequencing (HTS) is called metabarcoding and allows the multi-taxa identification, analysing the extracellular or total DNA extracted from samples that contain DNA of different origins [67]. The main advantage of metabarcoding is the capability to detect each single species in a complex herbal product and to elaborate mixtures simultaneously, where conventional analytical and DNA barcoding applications are limited [67].

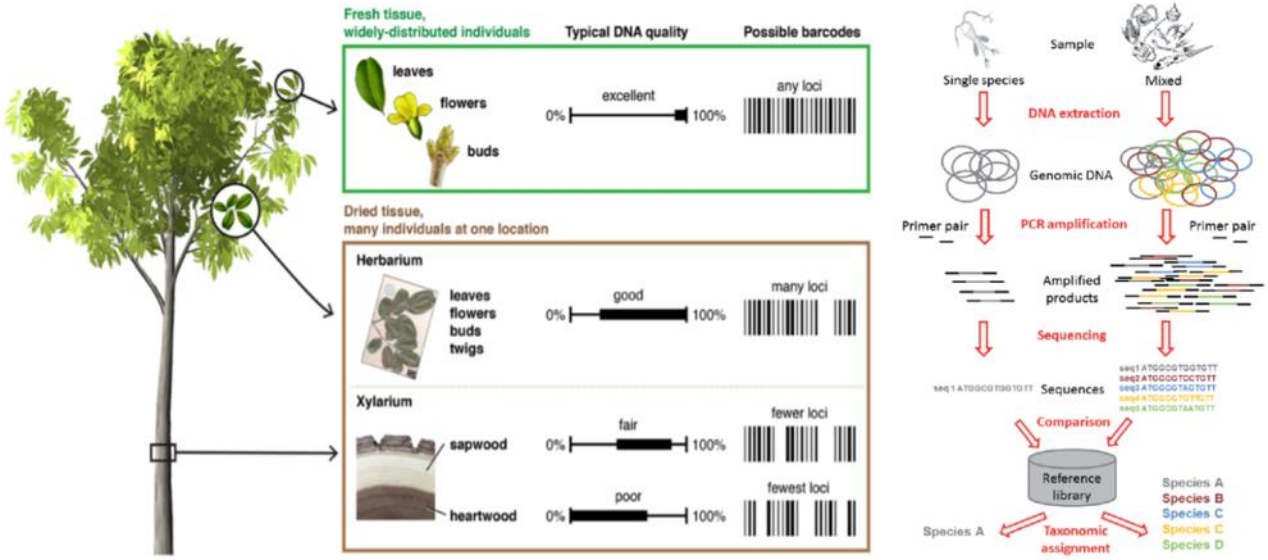


Fig. 10. Left; graphic representation of the potential strengths and weaknesses of source tissue in the development of DNA barcoding reference libraries [69]. Right; Representation of DNA barcoding (same genome) in the left, and metabarcoding (genomes of the species in analysis) in the right. After the sequencing of the amplified products, the taxonomic assignment is performed comparing the obtained sequences to those in a reference database [70].

The techniques, in a sample analysis, allow to detect the presence of contaminants and adulterations with other species. In quality control of herbal products, DNA barcoding and metabarcoding are applicable only in identification and authentication studies; in fact they do not provide any qualitative or quantitative information about compounds present in the plant material [64,71].

In animals, DNA barcoding is performed using sequences from the mitochondrial genome instead of the nuclear genome because recombination is rare and mitochondrial DNA is haploid, then it prevents errors during sequencing often caused by heteroplasmy [72]. In plants, the fragment of the cytochrome C oxidase (COI) mitochondrial gene has lower heterogeneity and is not suitable to distinct plant species. Therefore, DNA barcoding analysis is commonly performed on other DNA regions, in particular is possible to combine two or more genes to obtain more robust data. Some popular plant DNA barcodes are the nuclear internal transcribed spacer (*ITS*) region and the chloroplast intergenic spacer *psbA-trnH* and on two chloroplast gene fragments from the RuBisCo large subunit (*rbcL*) and maturase K (*matK*) genes [73]. In conclusion, the DNA barcoding of animals,

plants and other organisms will improve with the progress in polymerase chain reaction (PCR) amplification and DNA sequencing techniques. However, until the discrimination thresholds among species provides 100 % of accuracy, DNA barcoding techniques will be restricted. A significant future challenge is represented by adding more and more DNA information in order to enrich and complete genomic databases [63].

1.5. Punica granatum L.

1.5.1. Botany

Punica granatum L. (Lythraceae family) commonly known as pomegranate is a domesticated tree [74]. The genus *Punica* L. is characterized by two species: *P. granatum* and *P. protopunica* (theplantlist.org). The last mentioned is endemic in Socotra Island (Yemen) and is considered the ancestor of the genus *Punica* and the only relative of the cultivated pomegranate [75]. The taxonomic hierarchy of *Punica granatum* L. is shown in Table 4.

Table 4. Taxonomic hierarchy of *Punica granatum* L.

Division	Tracheophyta
Class	Magnoliopsida
Order	Myrtales
Family	Lythraceae
Genus	*Punica*
Species	*P. granatum*

It is widely grown as evergreen in the tropical regions and deciduous in temperate areas of the world. Botanically, pomegranate tree is a short shrub that has a bushy appearance, develops multiple trunks and it can grow up to more than 7 m when is not cultivated, while in dwarf cultivars do not exceed 1.5 m. It has shiny leaves (length 4-6 cm) and it makes red/orange bell shaped solitary flowers or organized in pairs or clusters. In the north part of the world, flowering occurs in April-May but in the young trees it may continue until the end of summer. Flowering and the resulting fruit last about 1 month. The red, spherical, globose or somewhat flattened fruits are approximately 10 cm in diameter. Each one has a prominent calyx that is a distinctive trait, and they contain numerous seeds covered by fleshy arils in the walls of membranous tissue. There are some exceptional cultivars such as the black pomegranate, characterize by black skin from very early stages of maturation until ripening time.

The edible part contributes to 55-60% of the whole fruit. Skin and arils colour ranges from light yellow to deep red, depending on the cultivar, but there is no correlation between the colours of these two. The best harvesting time, in general from 4.5 to 6 months after full bloom is influenced by the environmental and agronomic conditions. The fruit can reach its optimal nutrient and sugar composition, colour, size in Mediterranean-like climatic conditions which includes mild winters with temperatures not lower than -12°C and hot dry summers without heavy rainfall that can damage the fruit's development [76].
The pomegranate plant (Figure 11) requires moderate levels of fertilisers and grows better in slightly alkaline and well drained soils. It has a good drought tolerance, nevertheless a regular irrigation is important to reduce fruit disorders such as splitting [77]. The "husk" consists of two parts: the

pericarp, which is a cuticle layer; and the mesocarp (also known as the *albedo*), which is a spongy tissue where the arils are attached. Membranous walls (*septum*) and fleshy mesocarp separate multi-ovule chambers (*locules*) [78,79].

Fig. 11. A view of pomegranate tree with fruits [80]

1.5.2. Origin and cultivating regions

The name pomegranate is derived from the Latin of the fruit *Malum granatum*, that means ''grainy apple''. The term *Punica* indicates the Roman province of Phoenicia (Carthage), whence the Romans imported pomegranates. The term *granatum* is referred to the many seeds in its fruit. Despite pomegranate cultivation is considered as a minor crop, its use is an important part of the human history. Pomegranate tree is native to central Asia, its use began in prehistoric era and its domestication has been estimated in the Neolithic period.

Pomegranate is represented in various archaeological artefacts found in Egypt, Israel, Armenia and Mesopotamia, in addition carbonized fragments of pomegranate parts belonging to the early Bronze Age were found in the Middle East, an example is reported in Figure 12. The Phoenicians might have introduced its cultivation all over the Mediterranean region and Spanish sailors and Franciscan missionaries spread pomegranate cultivation in California and its near coastal regions in the 1700s. Nowadays it is cultivated in several geographical regions of the world including Asia, the Mediterranean area, South Africa, Australia, Argentine, Brazil and the United States due to its highly adaptive behaviour to a broad range of climates and soil conditions. [78,81–83].

Fig. 12. Ancient inscribed ivory pomegranate (Shanks, 2016)

1.5.3. The importance of pomegranate: state of the art

Nowadays, the correlation between food and drugs is becoming much clearer due to the ever improvements of scientific research. The term of nutraceutical was born 20 years ago to combine the words *nutrition* and *pharmaceutics*, both essential contributors of human wellness. In the last decades many studies were focalized to nutraceuticals, dietary supplements or functional foods, which comprise any food or food part that ensure healthy properties [84]. In this context, pomegranate, an ancient and beloved plant and fruit well known in folk medicine of different countries is becoming increasingly popular as a functional food and nutraceutical source in particular for its high polyphenols content, present not only in the edible part but also in other parts of the fruit and the plant such as peel, bark, leaves and flowers [85,86].

These findings allowed the public to become higher aware to the numerous healthy benefits provided at first by its fruit and juice particularly in the western world where recently their consumption is highly increased. In the last years as a result to the improvement of the industrial and agricultural techniques, the extent of pomegranate cultivations has been enlarged [78].
About 100 g of arils supply 83 kcal of energy, 1.67 g protein, 18.7 g carbohydrate, 3 mg sodium, 236 mg potassium, 12 mg magnesium, 10 mg calcium, 0.3 mg iron, 0.16 mg copper, 0.35 mg zinc, 12.2 mg vitamin C, 0.29 mg niacin, 38 µg folate and 16.4 µg vitamin K (USDA, 2019). Most of the scientific studies are focused on the edible parts of the fruit and its derived juice, and directed towards different cardiovascular risk factors, hypertension, diabetes, inflammation, antioxidant, antibacterial, antiviral and anticancer properties [87–92].

It has been shown that the consumption of pomegranate juice can give consistent health benefits through a significantly decreasing of systolic and diastolic blood pressure via an inhibition of ACE activity [93]. Studies on type 2 diabetic patients showed that pomegranate juice is an additional contribution in reducing blood glucose level [89]. Recent *in vitro* and *in vivo* studies correlate the presence of flavonoids, anthocyanins and hydrolysable ellagitannins to its vasculoprotective role, including a positive influence on the endothelial cell function, decreasing lipid uptake by macrophages and reducing platelet aggregation, lowering of oxidative stress and lipid oxidation. Lower hypertension and an attenuating atherosclerosis promoted by a daily intake of pomegranate juice have been demonstrated by human clinical studies. All these evidences suggest the potential helpfulness of wider pomegranate and its constituents used in a heart-healthy diet or as adjuvant in vascular diseases treatment [94]. A study conducted on aged man showed that pomegranate juice

has a powerful anti-inflammatory effect by reducing inflammation and muscle damage, explained by an inhibition of inflammatory markers such as NFkB, TNFα and COX-2 [95]. In addition, pomegranate juice and its purified polyphenol extract have been reported to have microbiocidal effects on HIV-1 and Influenza A virus, and showed a synergistic effect when associated with commercial antiviral drugs [91,96].

Among the scientific community the appreciation on dietary phytocompounds that represent beneficial agents and potential weapons in the prevention and fight against cancer has recently emerged because of their cost effective and safe nature. The presence of specialized bioactive compounds such as polyphenols in the pomegranate fruit has attracted the attention of the medical community. This chemical composition has been studied for its potential chemopreventive and cancer therapeutic effects in animal models [97].

Nowadays despite the good and promising results obtained from *in vitro* and *in vivo* studies, well designed clinical trials in human are still needed to consider the use of pomegranate as a novel strategy against cancer [98]. Despite the most studied pomegranate fruit and its derived juice, which are the most common way of its consumption, the non-edible parts of the plant deserve considerable importance. In pomegranate fruit peels, a principal waste by-product of the commercial fruit juice, and in the tree bark (heartwood), punicalagin α and β isomers (called punicalagins) are the most abundant hydrolysable tannins representing for over 85% of total tannins. Other hydrolysable tannins present in fruit peels, and heartwood are punicalin, gallagic acid, ellagic acid and ellagic acid glycosides [99–103].

The alcoholic extracts of pomegranate roots and bark have antibacterial activities and they may be used in mouthwash or toothpaste in the prevention or treatment of the oral cavity infections caused by *Candida* sp. [104]. Seeds oil extract is rich in punicic acid, an isomer of linolenic acid which can be helpful in the treatment of metabolic syndromes in humans [105]. In addition, it showed to have a significant anti-obesity effect and a neuroprotective potential by the inhibition of cholinesterase and the stimulation of the antioxidant capacity in brain [106].
Pomegranate peel and its derived extracts are suitable not only for pharmaceutical applications but also in the food industry as multi-functional preservative [107] and for cosmetical applications: in fact peel extract is able to stimulate keratinocyte proliferation and procollagen synthesis for facilitating skin repair [108].

Nowadays pomegranate extracts are used in several skin care commercial products. Other studies showed that pomegranate juice by-products such as seeds and peel which may cause environmental problems, can be also used for feeding ruminants and it represents a possible good nutritive supplement in ruminant diet by reducing cereal feeding [109]. Finally, pomegranate juice and seeds are used to make sauces and toppings to accomplish several type of dishes [110]. Figure 13 summarizes the main healthy benefits of pomegranate.

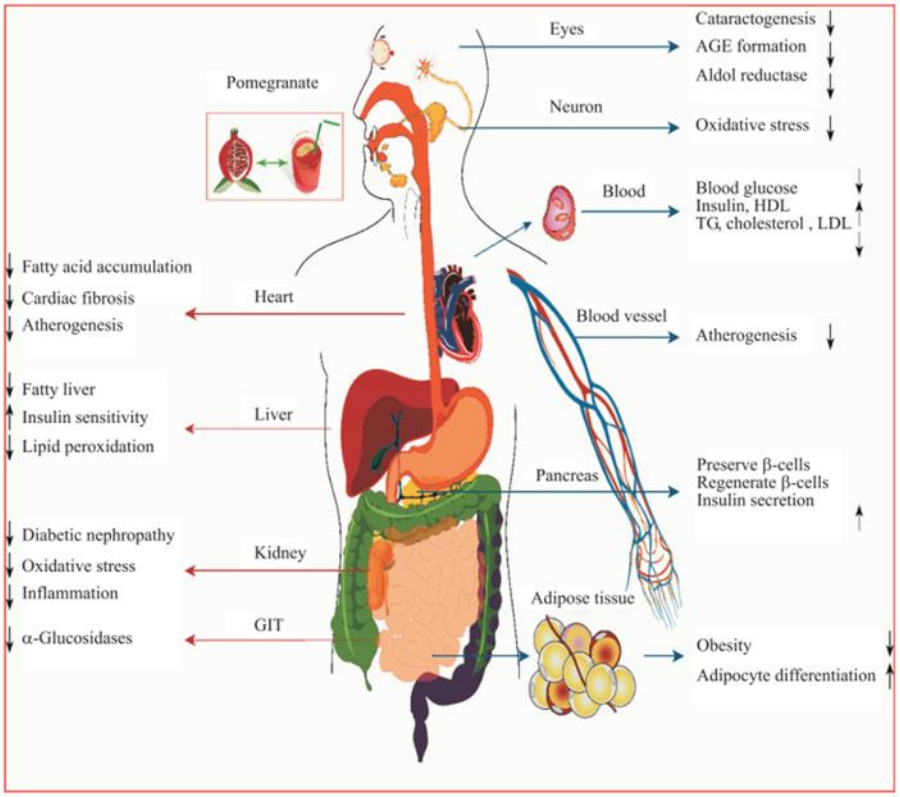


Fig. 13. Healthy properties of pomegranate and its by-products on human health [111].

1.6. *Vitis vinifera* L.

1.6.1. Botany

Vitis vinifera L., commonly known as grapevine, is a type of perennial defoliating vine belonging to the Vitaceae family. The Taxonomic hierarchy of *Vitis vinifera* L. is shown in Table 5. It is a wooden and often twisted stem which can reach up to 35 m long while in cultivation the length is reduced by the yearly pruning of the shoots and leaves to 1-3 m.

The leaves are thin, circular or oval, glabrous, with dentate or jagged margins with a diameter of 5-23 cm, basal sinus deep and lobes often overlapping, 5–7-lobed with a stormy gray tone on the lower face and a dull green colour on the upper face; tendrils are branched, normally opposite 2 leaves out of three. In autumn season, the leaves turn red because to the accumulation of anthocyanins mainly cyanidin-3-monoglucoside and peonidin-3-monoglucoside [112].
The flowers are numerous, pentamerous, fragrant, groped in clusters and arranged opposite to the leaves; the calyx is very shortly and five-lobed, petals are about 5 mm with pale green colour and sweet-scented.

The fruit consists in soft pulpy ellipsoid or globose berries called grapes of variable colours (from green, yellow to red or purplish-black) depending on the variety and is organised in large and long clusters. Berries are juicy, sweet or sour and contain 2-3 pyriform seeds with rather long beak [17]. All these parts of the plant are represented in Figure 14.

Fig. 14. Some *Vitis vinifera* organs: leaf (1), seed (2), grape (3), flower (4) (plantcurator.com).

Table 5. Taxonomic hierarchy of *Vitis vinifera* L.

Division	Tracheophyta
Class	Magnoliopsida
Order	Vitales
Family	Vitaceae
Genus	*Vitis*
Species	*V. vinifera*

1.6.2. Origin and cultivating regions

The term grapevine includes about sixty inter-fertile wild *Vitis* species located in North America, Asia and Europe under Mediterranean and continental–temperate climatic conditions [113]. Historically, grapevine has been used by people since ancient times and towards 5000 BC or even earlier, the cultivated European vine migrated from the north through Anatolia to Syria, thus to the Holy Land. Botanists attribute the origin of grapevine to an area extending from Southeast Europe to Western India.

Significant quantities of vinification residues (tartaric acid) with terebinth resin were found in clay jars, indicating evidences of ancient winemaking dated back to the end of the seventh millennium BC. From eastern Mediterranean areas, viticulture spread gradually westwards. The Phoenician seems to have played a significant role in the establishment and development of viticulture and viniculture. During the fifth millennium BC, grape cultivation started in Greece and Crete, whence numerous artefacts were found as Figure 15 represents an example.

Mentions of grapevine dated around 2500 BC were found in stone seals engraved depicting scenes of banquets with people drinking wine belonging to ancient Egyptian inscriptions [114]. Initially wild species were used and the subsequently domestication of the plant, allowed people to cultivate vine for fruit to be eaten fresh or dried, to make juice and wine. *Vitis vinifera* L. is the single *Vitis* species that obtained a significant long-term economic interest; some other species are used as breeding rootstock because of their resistance against grapevine pathogens [113].

Nowadays, grape crops represent one of the most important and widespread agro-economic activities around the world with the annual world production of more than 60 million tons dedicated to fresh consumption, juice and raisins. The large proportion of this production is devoted mostly to vinification processes, which primarily in Southwestern Europe constitute an important traditional activity [115].

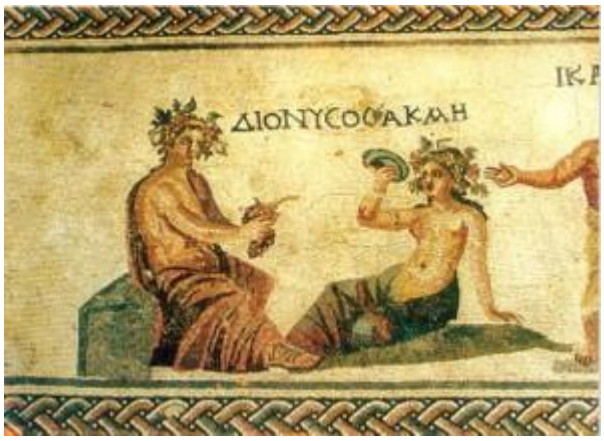


Fig. 15. Hellenistic mosaics discovered close to the city of Paphos depicting god of wine (Wikipedia.org).

1.6.3. Grapevine by-products

Unfortunately, huge amounts of residues are generated every year from viticulture and wine production processes. These residues can cause environmental problems when they are discarded in open areas for their considerably high biochemical and chemical oxygen demands [116,117]. In order to potentiate the "by-product" market limiting the production of waste and to reduce waste on landfill sites, the Directive 1999/31/EC and subsequently Directive 2008/98/EC establish the basic waste management concepts, such as the definitions of waste, recycling, reuse and recovery (eur-lex.europa.eu). The latter explains when waste can be considered as secondary raw material as well as the differences between waste and by-products. The latter is a substance or object obtained from a production process, but it does not represent the primary goal of the production being a by-product only if it possess the following conditions: 1) a certain further use of the substance or object; 2) over the normal industrial practice, the substance or object can be used directly without any further processing; 3) on a production process, the substance or object is

produced as an integral part of it; 4) lawful use i.e.: the substance or object will not lead to overall adverse environmental or human health impacts.

By-products generated by viticulture and wine production are grape pomace (mainly skins and seeds), stems and leaves. Nowadays there is an increasing demand for green materials and renewable sources of nutrients and bioactive compounds; at the same time to reduce the environmental impact of agricultural and winery activities, their valorisation is of great interest for the feed/food, pharmaceutical and cosmetic fields. Grape pomace originates when grapes are pressed during the production of must and represents the most widely studied by-product. Its production, around 9 million tons per year, is on average 20% (w/w) of the total grapes used for winery industry per year [35,120,121]. This material is suitable for the extraction of grape seed oil and polyphenols such as phenolic acids, catechin, epicatechin, tannins, flavonoids and resveratrol, for the production of ethanol, methanol, citric acid, xanthan gum and energy by methanization via fermentation and distillation processes [122].

The anthocyanins extracted from grape skins and responsible for their colour, are used as natural food colorant with the EU number E163 [123]. Grape stems, removed before vinification procedure to avoid negative organoleptic characteristics to the wine, represent another by-product and they are used mainly as animal feed or soil amelioration.

In Asia, Eastern Europe and in the Mediterranean area, young grapevine leaves are edible and used in cooking, wrapped around fish or meat and then baked. In the Mediterranean Basin, the leaves and shoots produced by the annual pruning of vineyards after the grape harvest are eaten by sheeps and goats [125]. Despite these residues contain high levels of lignin and condensed tannins, vine leaves are a safe fodder without any toxic effect [126]. The main issue is represented by the use of pesticides for the treatment of grapevine; leaves spread with these products could cause toxicity and should be kept away from animals [127].

1.6.4. Applications of grapevine and its by-products for human health

Grapevine fruit contains various phenolic compounds, flavonoids and stilbenes. Nowadays, bioactive constituents found in the grape fruits, seeds, stems, skin and pomaces have been identified and studied [128]. All these parts are known to contain phytochemicals with health-promoting benefits suitable for pharmacological, cosmetic and food applications. Literature reports their valuable activity for the prevention of cardiovascular and degenerative diseases, oxidative reactions and inflammatory processes in humans [118,119].

Due to its nutritional value and many beneficial properties, the grape is an interesting edible food. Composition of the fruit is very complex and varies with the cultivating region, cultivar, climate conditions and degree of maturation [129]. Fresh grapes consist in a rich source of water (82%), carbohydrates (12–18%), proteins (0.5–0.6%) and fat (0.3–0.4%). Moreover, grape represents a good source of boron, potassium (0.1–0.2%), calcium (0.01–0.02%), phosphorus (0.08–0.01%), vitamin C (0.01–0.02%) and vitamin A (0.001–0.0015%) [130].

The most representing medicinal properties of grape constituents are: antidiabetic, antioxidant, anti-atherogenic, neuroprotective, anti-aging, anti-infection, anti-obesity, antimicrobial, anticarcinogenic and immunomodulatory [17,130,131]. Studies on grape juice and seed extracts

from black table grapes, showed their strong inhibitory effect against the growth of *Listeria monocytogenes* [132]. In particular, many biological activities has been attributed to resveratrol, a major compound extracted from the grape skin and seeds [133,134].

Grapevine seed extract is also used as anti-caries agent, oral care agent, oral health care drug, anti-dandruff, anti-fungal, anti-microbial, flavouring, antioxidant, light stabilizer and sunscreen agent. According to the Voluntary Cosmetic Registration Program (VCRP), grapevine leaf extract is used in 78 cosmetic formulations [135]. Since the polyphenols are capable of absorbing UV and visible light, the dried leaf extract of *Vitis vinifera* and *Vitis labrusca* was used to developed a phytocosmetic: a topical formulation for skin protection against ultraviolet radiation damage [136]. Nowadays, wine is used for healing and beauty purposes at several beauty centres worldwide. The ''wine therapy'' treatments combine hydrothermal techniques with grape-derived products offering a remarkable relaxing effect as well as muscular tonification and improved blood circulation [137].

The therapeutic potential of grapevine and its by-products have been applied for ages. Grapes have been used to prevent or treat pathological conditions such as nausea, gastroenteritis, diarrhoea and skin disorders [138]. In addition, wine has been reported to improve endothelial dysfunction, blood lipid parameters, platelet aggregation and other risk factors for cardiovascular diseases [139]. Grape leaves are the least studied and valorised residue; they represent a promising source of compounds with biological potential for human health [122,124]. They were used in the past to relieve pain, inflammation, diarrhoea, to stop bleeding and to drain furuncles [140] and studies on their extract showed antiviral and antioxidant activities supporting the utilization of grapevine leaves in folk medicine [141]. Figure 16 summarizes the principal healthy benefits of grapevine in the human body. Their beneficial properties are attributable to the polyphenols they contain [138,142].

Preclinical studies attributed to the leaf extracts the capacity to prevent or attenuate the severity of liver diseases induced by oxidative stress; to decrease lipid and protein oxidative damage induced by hydrogen peroxide in the rat brain, restoring the SOD and CAT activity; to impair the NFkB pathway, related to transcriptional control of pro-inflammatory mediators *in vitro* in a model of human gastric and intestinal epithelial cells; to exert beneficial effects *in vivo* on the kidneys of diabetic rats [143].

In conclusion, grapevine leaves give advantages over other viticulture by-products and possess an antioxidant potential 10 times higher than grape pulp or juice without containing alcohol or sugar; moreover they represent an inexhaustible and a low cost resource coming from the annual pruning of the vineyards [143].

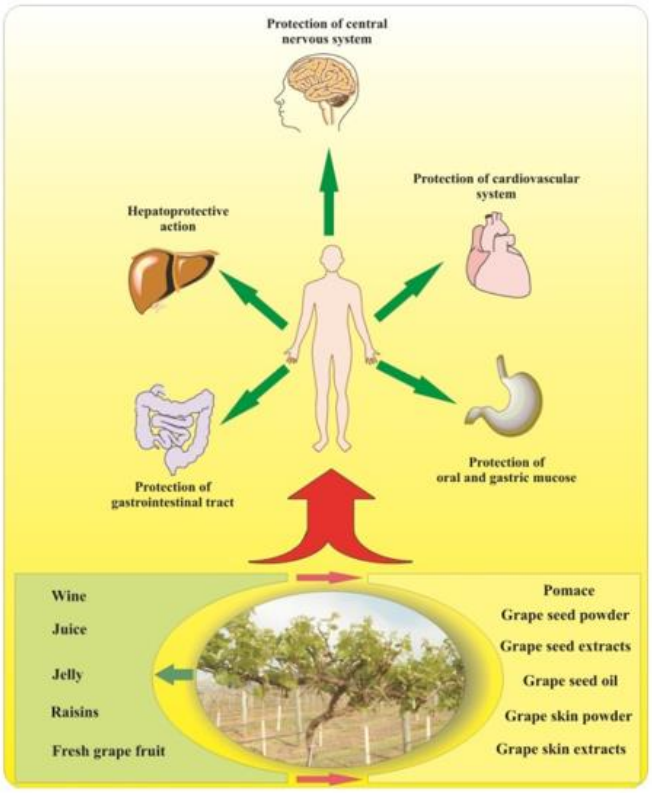


Fig. 16. Principal healthy benefits exerted by primary grape products and by-products on human body [131].

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Chapter 2

Phytochemical and biomolecular characterization of the non-edible parts of pomegranate

CHAPTER 2: PHYTOCHEMICAL AND BIOMOLECULAR CHARACTERIZATION OF THE NON-EDIBLE PARTS OF POMEGRANATE

2.1. Introduction and preliminary studies

As mentioned in Chapter 1, recent studies supported the traditional uses of all parts of pomegranate plant, including non-edible parts such as bark, peel and leaves for the potential beneficial properties [1]. Therefore in this PhD project, pomegranate leaf were chosen as a raw material to be studied both for the positive results in some biological tests [2,3] and because literature currently reports little information compared to other edible and non-edible parts of pomegranate; moreover, leaves' collection is a sustainable approach as it doesn't cause damage to the plant during the spring pruning or in the fall season. The leaves have been used in natural and holistic medicine to treat a broad range of diseases such as sore throat, cough, digestive disorders, urinary infections, conjunctivitis and diarrhea [4,5].

Recent research studies indicate the pomegranate leaf as an important source of bioactive secondary metabolites and the healthiest constituents are ellagic acid, ellagitannins, flavonoids, anthocyanins, estrogenic flavonols, flavones, saponins, carotenoids and terpenoids. The presence of these compounds may be associated to the studies related to a broad range of biological properties such as *in vitro* antioxidant, anti-inflammatory, anti-cholinesterase and anti-proliferative activities, where the ethanolic extract represents one of the most active [3,6,7]. The high polyphenolic content and all these important biological activities showed that pomegranate leaf could be valuable for applications in food, cosmetic and pharmaceutical industry as a potential source of active molecules. Based on the above considerations, this study started in collaboration with the Department of Life and Environmental Sciences of the University of Cagliari, and the first aim was to investigate the phytochemical composition of the pomegranate peel, bark and leaf ethanolic extracts; these non-edible parts showed anti-viral activity in preliminary *in vitro* assays by the inhibition of two HIV-1 enzymes: reverse transcriptase-associated RNase H activity and integrase (Table 1).

Table 1. Results of the *in vitro* preliminary assays of the pomegranate peel, bark and leaf ethanolic extracts on HIV-1 reverse transcriptase (RT) and integrase (IN) compared to commercial drugs as control.

Pomegranate ethanolic extracts and controls	HIV-1 RT RNase H IC50 (µg/ml)	HIV-1 IN LEDGF dependent IC50 (µg/ml)
Bark	0.22 ± 0.04	0.18 ± 0.02
Peel	0.85 ± 0.01	0.5 ± 0.04
Leaf	0.61 ± 0.02	0.12 ± 0.06
Efavirenz	0.02 ± 0.005	-
Raltegravir	-	0.06 ± 0.01

They are key functional enzymes for the replication and the integration of HIV-1 viral genome into the host chromosome and represent two of three major target in the HIV-1 treatments [8]. On the basis of the preliminary results, leaves were more in depth investigated from a phytochemical point of view to evaluate their contents in terms of specialised metabolites and to verify the potential variability of the extracts; these information are fundamental for the reliability of the biological

studies (Chapter 3); for this reason the phytochemical pattern was evaluated for plants of different geographical origin, harvesting season and year. Samples were collected from several Sardinian locations and occasionally from other regions. The phenolic composition of pomegranate leaf was determined through HPLC-PDA-ESI-MS/MS analysis after their ethanolic extraction. Together with the phenolic fraction, the ethanolic extracts reveal the presence of a triterpenoid fraction, whose characterization were run through the derivatization of the samples and GC-MS analysis. The quantitation of the main compounds was performed using the external standard calibration method and statistical data elaborations were adopted to highlight differences and similarities among the samples.

Moreover, the phenotypic differences were correlated to the biomolecular analysis with the evaluation of different DNA regions of the leaf samples collected in Sardinia and in other sites; this part of the study was in cooperation with the Plant Physiology Unit (Department of Life Sciences and Systems biology) of the University of Turin. The analysis of some DNA barcoding genes has been applied with success for discrimination of plant and animal samples. It may represent a useful and additional tool for the authentication of vegetal raw materials, in particular when comparable species show a similar chemical composition. A limit of this method is the usually absence in the reference databases of sequences of uncommon plants [9,10]. In this project two barcoding genes, the *ITS* and *psbA-trnH* were investigated to verify the stability of pomegranate from different harvesting sites.

2.2. Materials and methods

2.2.1. Plant materials

Leaves were collected from different sites in Sardinia from June to October in 2017 and 2018. More leaf samples were collected occasionally in other regions as Piedmont and Lombardy and one sample in Greece (Table 1). Sample 8 (PGL8) was from the botanical garden of the University of Cagliari, Italy, where a voucher specimen (CAG 514/A) was deposited. All individuals sampled from other sites were collected randomly. The fresh plant materials were dried at 40°C to constant weight.

Table 2. Location, coordinates, and code of pomegranate leaf samples.

Location	Coordinates	Code
SARDINIA (Museddu)	39°46'46.9"N 9°39'41.3"E	PGL1
SARDINIA (Museddu)	39°46'32.9"N 9°38'49.6"E	PGL2
SARDINIA (Pelau)	39°46'18.2"N 9°38'16.0"E	PGL3
SARDINIA (Sa Canna)	39°45'01.4"N 9°34'28.2"E	PGL4
SARDINIA (Jerzu)	39°47'47.1"N 9°31'08.9"E	PGL5

SARDINIA (Jerzu)	39°47'45.6"N 9°31'08.5"E	PGL6
SARDINIA (Gairo Taquisara)	39°51'26.5"N 9°27'51.5"E	PGL7
SARDINIA (Cagliari botanical garden)	39°13'19.6"N 9°06'42.6"E	PGL8
LOMBARDY (Brescia)	45°32'20"N 10°13'13"E	PGL9
PIEDMONT (Cambiano)	44°58'00"N 7°47'00"E	PGL10
GREECE (Mykonos)	37°27'N 25°20'E	PGL11
PIEDMONT (Biella)	45°33'59"N 8°03'12"E	PGL12

2.2.2. Chemicals

HPLC-grade acetonitrile (LC-MS grade), methanol, Pyridine, N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) , formic acid (>98% purity), ellagic acid, rutin, apigenin, were purchased from Merck (Milan, Italy). De-ionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Luteolin, apigenin 7-*O*-glucoside, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 4'-*O*-glucoside, betulinic acid, oleanolic acid, ursolic acid, punicalin α+β, punicalagin α and punicalagin β were purchased from Extrasynthese (Genay Cedex, France).

2.2.3. Sample preparation

From each pomegranate leaf sample, two extracts were prepared. 0.500 g of dried and ground powder was extracted with an ultrasonic bath (Soltec, Sonica S3 EP 2400) operating at 40 KHz with 10 mL of ethanol, three times for 10 min. The supernatants were then combined and centrifuged at 5000 rpm for 10 min and poured into a glass ballon and evapored in a rotary evaporator under vacuum at a temperature below 50°. To reduce chlorophyll interference 30 mg of crude extract were resuspended in 1 mL of methanol/water (20:80, v/v), loaded on Bond Elut SPE-C18 cartridge (Agilent Technologies, USA) and eluted with 8 mL of methanol/water (95:5, v/v) and then evaporated in a rotary evaporator. For each extract a 10 mg/mL stock solution in methanol, was prepared and it was subsequently diluited to 2.5 mg/mL using acetonitrile/water (95:5, v/v) and filtered through a 13 mm diameter, 0.22 µm PTFE syringe hydrophilic filter before the HPLC-PDA-MS/MS analysis.

2.2.4. HPLC-PDA-MS/MS analysis

2.2.4.1. Qualitative analysis

Each extract (5 µL) was analysed with a Shimadzu Nexera X2 system equipped with a photodiode array detector SPD-M20A in series to a Shimadzu LCMS-8040 triple quadrupole system provided with an electrospray ionization (ESI) source (Shimadzu, Dusseldorf Germany). Samples were analysed with an Ascentis Express RP-Amide column (10 cm ×2.1 mm, 2.7 µm, Supelco, Bellefonte, USA) using water/formic acid (999:1, v/v) and acetonitrile/formic acid (999:1, v/v) as mobile phases

A and B, respectively. The flow rate was 0.4 mL/min and the column temperature was maintained at 30 °C. The gradient program was as follows: 5% B for 5 min, 5–25% B in 35 min, 25–100% B in 10 min, 100% B for 1 min. Total pre-running and post-running time was 56 min. UV spectra were acquired over the 220-450 nm wavelength range. Mass spectrometer operative conditions were: heat block temperature: 200 °C; desolvation line (DL) temperature: 230 °C; nebulizer gas (N_2) flow rate: 3 L/min; drying gas (N_2) flow rate: 15 L/min. Mass spectra were acquired both in positive and in negative full-scan mode, over the range 50–1500 m/z, event time 0.5 s. Product Ion Scan mode (collision energy: - 35.0 V for ESI+ and 35.0 V for ESI−, event time: 0.2 s) was applied to compounds for which the pseudomolecular ions $[M+H]^+$ in ESI+ and/or $[M-H]^-$ in ESI− were identified. Selected Reaction Monitoring acquisition (collision energy: - 35.0 V for ESI+, dwell time: 20) was performed on specific product ions derived from precursor ion fragmentation. Some of the main components were identified by comparing their retention times, UV and MS spectra with those of authentic standards. The other components were tentatively identified through their UV spectra and mass spectral information, compared to those reported in the literature.

2.2.4.2. Quantitative analysis

Quantitation was performed by the external standard calibration method, and the results expressed as mg of compound per g of matrix (mg/g). The calibration curves of luteolin 4'-*O*-glucoside and ellagic acid were built up by analyzing them at eight concentrations in the range 0.1-100 mg/L, while for punicalin α+β mixture, five concentrations in the range 5-100 mg/L and for both punicalagin α and punicalagin β, five concentration in the range 10-500 mg/L were made; all compounds were dissolved in methanol and used PDA detector set at the λ max for each compound, while for luteolin 7-*O*-glucoside, rutin, quercetin 3-*O*-glucoside and apigenin 7-*O*-glucoside five concentrations in the range 0.1-10 mg/L and for apigenin and luteolin in the range 0.1-5 mg/L were used and they were quantified using the selected reaction monitoring in ESI^+ mode because of coelutions. In this case the transitions from the product ion scan analysis for each standard compounds were adopted as reported in Table 2. For those components whose the commercial standard compound was not available, the calibration curves built with PDA of compounds belonging to the same chemical class were used. The analytical performances were measured in terms of repeatability (RSD% never exceeding 5%) and intermediate precision (RSD% never exceeding 15%). All data were processed using LabSolution software (Shimadzu, Dusseldorf Germany).

Table 3. Quantification method, linearity range, R^2 and calibration curve of the main compounds in LC-MS.

Compound	Method	Linearity range (μg/mL)	R^2	Calibration curve
Luteolin 4'-*O*-glucoside	UV 340 nm	0.1-100	0.999	y = 44443.1x – 8761.6
Ellagic acid	UV 370 nm	0.1-100	0.999	y = 44443.1x - 8,761.63
Rutin	SRM^+ 611→303	0.1-10	0.999	y = 1523640.1x + 82583.6
Luteolin 7-*O*-glucoside	SRM^+ 449→287	0.1-10	0.999	y = 3182557x + 1145226
Quercetin 3-*O*-glucoside	SRM^+ 465→303	0.1-10	0.999	y = 1300399.6x + 186468.5

Apigenin 7-*O*-glucoside	SRM $^{+}$ 433→271	0.1-10	0.998	y = 5407146.2x + 866368.8
Luteolin	SRM^{+} 287→153	0.1-5	0.999	y = 1181554.7x + 61020.8
Apigenin	SRM^{+} 271→153	0.1-5	0.999	y = 1072063.8x + 545489.2
Punicalin α+β	UV 370 nm	5-100	0.999	y = 5759.8x – 9501.1
Punicalagin α	UV 370 nm	10-500	0.999	y = 1834.8x – 11147
Punicalagin β	UV 370 nm	10-500	0.998	y = 2425.9x – 1031

2.2.5. GC-MS analysis

2.2.5.1 Qualitative analysis

GC analysis were carried out on a Shimadzu 2010 GC unit coupled to a Shimadzu QP2010 Mass spectrometer using a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany). Pyridine (80 ul) and BSTFA (120 ul) were added to 2 mg of each crude extracts and heated at 60 °C for 30 min to turn hydroxylated and carboxylic components into the corresponding trimethylsilyl derivatives (TMS). GC-MS analysis were carried out on MEGA-1 column (100% Methyl Polysiloxane, 15 m x 0.18 mm x 0.18 µm) from MEGA S.r.l. (Milan, Italy). Analytical conditions: injector temperature: 300°C, transfer line temperature: 320°C; ionization energy: 70 eV, carrier gas: He (0.8 ml/min), split ratio 1:10. The oven temperature was programmed rising from 50°C (2° min) to 300°C at 5°C/min. Total run time for each sample was 52 min. MS conditions were: source temperature: 230°C; quadrupole temperature: 150°C. The MS operated in electron impact ionization mode at 70 eV, at a scan rate of 0.2 µ/s with a mass range of 100–650 m/z; Volatile components were identified by comparison of mass spectra and their linear retention indices (ITs), calculated versus a C9-C25 hydrocarbon mixture with those reported in literature [16]; in particular the identity of the triterpenoids was also confirmed by the co-injection of commercially available standards.

2.2.5.2. Quantitative analysis

Quantitation of triterpenoids was performed through external standard calibration method. Suitable amounts of oleanolic acid and ursolic acid were diluted with acetone to obtain seven concentrations in the range 1 to 250 µg/mL for each marker and for betulinic acid six concentrations in the range 1-125 µg/mL were used. 1 ml of standard solution for each concentration was evaporated to dryness and derivatized to the corresponding TMS. Each concentration was derivatized in duplicate. Calibration curves were obtained by analyzing the resulting TMS-derivatized standard solutions two times by GC-MS in SIM acquisition mode under the previously concentrations. Target and qualifier ions for SIM acquisition mode were: 189; 203; 320 m/z for the

triterpenic compounds. Data were processed using Shimadzu GCMS Solution software (Shimadzu, Dusseldorf Germany).

Table 4. Quantification method, linearity range, R^2 and calibration curve of triterpenoids in GC-MS.

Compound	Target ion	Qualifier ions	Linearity range (µg/mL)	R^2	Calibration curve
Oleanolic acid	203	189, 320	1-250	0.995	y = 33844x - 303001
Betulinic acid	189	203, 320	1-125	0.989	y = 13031x – 87256
Ursolic acid	203	189, 320	1-250	0.995	y = 31162x - 287048

2.2.6. DNA extraction, PCR amplification and sequencing

Ten mg of each pomegranate leaf samples were ground to a fine powder and mixed with 5mg of polyvinylpolypyrrolidone (PVPP, Sigma Aldrich, Bellefonte, USA)., Genomic DNA was isolated from the ground fine powder using the Nucleospin Plant II Kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. The qualitative and quantitative spectrophotometric analysis of the isolated genomic DNA were performed using a Nanophotometer (Implen GmbH, Munich, Germany) and 1% (w/v) agarose gel electrophoresis (visualized by ethidium bromide staining under UV). About 20 ng of genomic DNA were used as a template for PCR amplification, forward and reverse primers specific for the amplification of the *ITS* and *psbA-trnH* regions were used (Table 5). Amplification was performed in a 25 µL reaction mixture containing 0.2 mM deoxynucleotide triphosphates (dNTPs), 20 pmol of forward and reverse primers, 0.5 U of Taq DNA polymerase (ThermoScientific, Waltham, MA USA) and 2.5µL of 10x PCR buffer (Thermo-Scientific, Waltham, MA USA). PCR reactions were performed in a Temperature Gradient Thermalcycler (Biometra, Jena, Germany). Cycling conditions consisted of an initial 4 min at 94°C, followed by 30s denaturing at 94°C, 45s annealing, respectively at 53°and 56°C for *ITS* and *psbA-trnH*, and 45s elongation 72°C, repeated for 35 cycles and with 10 min final extension at 72°C. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. PCR products were employed as a template for sequencing (Eurofins Genomics, Vimodrone (MI), Italy). Both DNA strands were sequenced.

Table 5. List of primers used in PCR and sequencing.

Primers	Primers sequences	Tm [°C]	References
***ITS* 1**	TCCGTAGGTGAACCTGCGG	61	[17]
***ITS* 4**	TCCTCCGCTTATTGATATGC	55.3	[17]
***psbA* Forward**	GTTATGCATGAACGTAATGCTC	56.5	[18]
***trnHf*-05**	CGCGCATGGTGGATTCACAATCC	64.2	[19]

2.2.7. Statistical data treatment

All analyses were performed in duplicate and data were expressed as mean values ± standard deviation. All statistical elaboration (Principal Component Analysis, Box Plots and Student's t-test) were carried out using Statistica 10 (StatSoft. Inc., Tulsa, OK, USA) and SPSS 15.0 (IBM Corporation) softwares. Principal Component Analysis (PCA) was performed in order to reduce the multivariate space in which items are distributed and to verify similarities and dissimilarities between the investigated samples and Box Plots and Student's t-test allowed to define statistical differences concerning compound abundances (P<0.05). The sequencing quality of the gene sequences were initially checked with Chromas software (Technelysium Pty Ltd.) and subsequently the sequences were aligned with CLC sequence viewer software (Quiagen®) using default parameters to check the integrity of each sample sequence. Consensus sequences obtained from the alignment of the individual sequences of each sample analysed were compared with those deposited in literature (BLAST-NCBI).

2.3. Results and discussion

2.3.1. Phytochemical analysis using HPLC-PDA-MS/MS and GC-FID-MS analytical platforms

Liquid chromatography coupled to mass spectrometry particularly HPLC-PDA-MS/MS is a robust analytical technique for the analysis of complex systems [20]. Gas chromatography (GC) (conventional, fast or enantioselective) combined with FID or MS detector, is a common analytical approach, with high speed, precision, and selectivity, used to analyse volatiles or compounds that can be vaporized without decomposition in a complex matrix [21,22]. In this project they have been used to separate and identify the main specialized metabolites found in pomegranate extracts considering also the literature data [22–26].

2.3.1.1. Phytochemical analysis of bark and peel ethanolic extracts

At first, the specialized metabolites present in pomegranate bark, peel and leaf ethanolic extracts resulted active on the *in vitro* inhibition of two HIV-1 key functional enzymes in preliminary assay as reported in the paragraph 2.1 were characterized by HPLC-PDA-MS/MS analysis. In order to optimize the separation in terms of resolution of analytes and analysis time, the stationary phase and the gradient program were studied. The optimization of the chromatographic separation of the compounds was investigated on three based reverse phase columns: Ascentis® Express C18 (15cm x 2.1 mm, 2.7 µm), Phenyl-Hexyl (10cm x 2.1 mm, 2.7 µm) and Amide (10 cm ×2.1 mm, 2.7 µm). A fused-core RP-Amide column as stationary phase provided an increase in separation selectivity and represented the best solution to simplify the phytochemical complexity of pomegranate extracts. The best conditions in terms of resolution and time of analysis were obtained using a gradient elution mode with an acidified acetonitrile-water mixture as a mobile phase with formic acid 0.1%, (see paragraph 2.2 Materials and methods).

In a classical alkyl-silica (C18) stationary phase, the interactions between analytes and silanol groups can occur, resulting in increased retention and poor peak shape. The incorporation of an embedded polar group (EPG) into the bonded alkyl chain was developed to minimize the interactions with these

silanol group on the silica surface of reversed phase columns. This suppression of silanols can be due to the hydrogen bonds among the residual silanol groups and a water layer around the EPG or directly with the EPGs. RP-Amide column is a more polar embedded stationary phase lately developed whose properties allow the use as an eluent up to 100% water. The possibility of working in 100% water conditions and the improved selectivity and retention for polar compounds make this column an effective tool for the HPLC analysis of polyphenols and derivatives. RP-Amide has enhanced dipole-type interactions and better interactions with lone pair and p-electrons donor solutes. Compared to classical C_{18} columns, these characteristics allow to obtain lower hydrophobicity and hydrogen-bond donor ability, higher hydrogen-bond acceptor ability, the increase of the retention and selectivity for polar compounds [27–29].

Thanks to the use of an RP-Amide column coupled with in tandem mass spectrometer detector, the analytical method for analysing polyphenols significantly increased selectivity and sensitivity to detect and quantify the main components of non-edible parts of pomegranate and in the case of leaves this study gives an interesting contribution with respect to the current literature [23,30,31].

Figure 1A and 1B reported as an example, the chromatograms of pomegranate bark acquired with a RP-C_{18} and RP-amide column, respectively. It is possible to observe the numerous peak coelutions by using the RP-C_{18} column in comparison with the better resolution obtained with the RP-amide in the separation of the compounds of the crude extract.

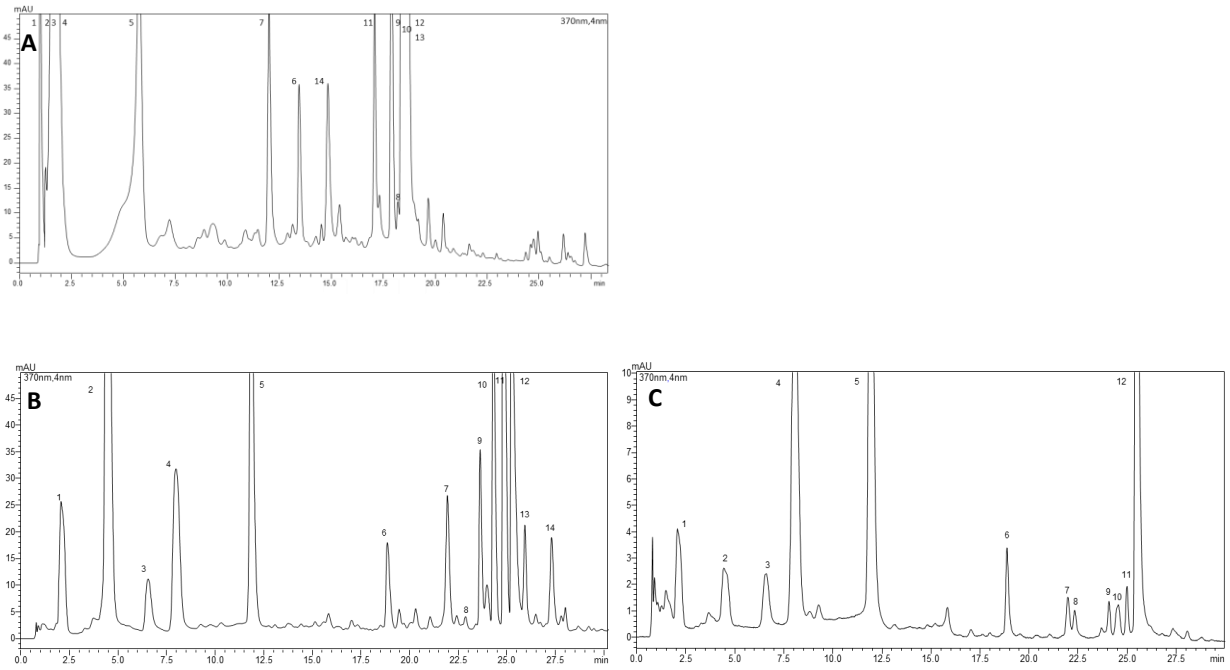

Fig. 1. LC representative chromatograms of the pomegranate non-edible parts. Compounds are numbered according to Table 6. (A, B) Bark extract separated by using RP-C_{18} and RP-amide column respectively and (C) peel extract separated with the RP-amide column.

The identification of metabolites in pomegranate bark, peel, and leaf ethanolic extracts was obtained by combining the information from PDA, MS and MS/MS and comparing them with the literature data [25,32–36].

Phenolic compounds belonging to different classes were detected and when possible, their identification was confirmed by the co-injection of reference commercial standards. Table 6 reports the main compounds identified in bark and peel extracts. The UV–vis chromatograms on RP-amide

column of the two extracts at wavelength of 370 nm are shown in Figure 1B-1C. UV-vis spectra were used to distinguish polyphenols. In particular, ellagitannins are characterized by two major absorbtion peaks , the first one in the region 250-259 nm (defined as Band II) and the second in the region 360–380 nm (defined as Band I) [32]. MS spectra were acquired in total ion scan to obtain pseudomolecular ions both in positive and in negative scan mode and in product ion scan on the pseudomolecular ions to obtain diagnostic fragments. According with the existing literature, bark and peel extracts revealed the ellagitannins as the most representative compounds. In particular large hydrolyzable tannins such as punicalins and two punicalagin anomers (α and β) were found [34]. Anthocyanins were below the limit of detection in peel extract sample.

Table 6. List of identified and putatively identified compounds in bark and peel extracts. Each compound is referred through its relative retention time, UV spectrum (λ are mentioned in order of maximum λ absorption), molecular formula, pseudomolecular ions (ESI^+ and ESI^-), molecular weight fragments generated by product ion scan mode (PIS) and identified or tentatively identified compound names. The identification confidence value and the literature reference that indicates the presence of the compounds in pomegranate are also reported.

N°	RT	λmax (nm)	Molecular formula	$[M + H]^+$	$[M-H]^-$	Supposed MW	MS^{2+} m/z	MS^{2-} m/z	Compound name	Identification confidence[b]	Ref.
1[a]	2.076	257 377	$C_{34}H_{22}O_{22}$	783	781	782	603	601	Punicalin isomer	1	[36]
2	4.414	258 376	$C_{48}H_{28}O_{30}$	1085	1083	1084	603	601	Punicalagin isomer	2	[37]
3	6.553	257 370	$C_{21}H_{10}O_{13}$	471	469	470	453 407 151 363	451 425 353 341	Valoneic acid dilactone	2	[38]
4[a]	7.984	257 376	$C_{48}H_{28}O_{30}$	1085	541 (+1083)	1084	621 603	601	Punicalagin α	1	[36]
5[a]	11.853	257 380	$C_{48}H_{28}O_{30}$	1085	541 (+1083)	1084	765 621 603	781 721 301	Punicalagin β	1	[36]
6	18.842	252 360	$C_{20}H_{16}O_{13}$	465	463 (+927)	464	345 315 303 285 223	301 283	Ellagic acid glucoside	2	[39]
7	21.931	254 364		471	469	470	407 303 168 139	301 271 227 201 171	Sanguisorbic acid dilactone	2	[34]
8	22.859	252 364		435	433	434	303 285 275	301	Ellagic acid derivative	3	
9	23.613	251 360	$C_{19}H_{14}O_{12}$	435	433	434	303 285	301 283	Ellagic acid-pentoside	2	[36]
10	24.279	251 360	$C_{20}H_{16}O_{12}$	449	447	448	303 285 273	301 257 229	Ellagic acid-deoxyhexoside	2	[39]
11	25.409	255 373		1067	1065 (+532)	1066	603 575	/	Ellagitannin	3	
12[a]	25.658	252 366	$C_{14}H_6O_8$	303	301	302	/	284 229 185	Ellagic acid	1	[36]
13	25.934	255 372			301 (+603)			275 256 127	Ellagitannin	3	

14	27.309	256 379			601			101	Gallagic acid dilactone	2	[34]

[a] Compounds identified by comparing with reference standards.

[b] An identification confidence according to the request of the CAWG (2007) is indicated: Level 1:Identified compound (A minimum of two independent orthogonal data (such as retention time and mass spectrum) compared directly to an authentic reference standard; Level 2:Putatively annotated compound (Compound identified by analysis of spectral data and similarity to bibliographic data); Level 3: Putatively characterised class compound.

In bark and peel extracts, punicalins, punicalagin α and β and ellagic acid were confirmed by the co-injection of authentic commercially available standards, while the other components were putatively identified by comparison of their UV spectra and mass spectrometry fragmentation pattern with those reported in literature (Table 6). For instance, ellagic acid glucoside was tentatively identified by its UV spectral data (UV_{max}= 252/360nm), pseudomolecular ions 465 m/z and 463 m/z, in ESI^+ and ESI^- ionization modes, respectively, and with PIS to give diagnostic ion at 301 m/z in ESI^- [33,39]. Compound 6 and 9 were identified as ellagic acid glucoside and pentoside due to molecular weight of their glycosyl group: 162 and 132 g/mol, respectively and corresponding to glucose and pentose sugar after a water molecule loss [36,39].

In conclusion the phytochemical analysis of bark and peel extract reveals the importance of the tannin fraction composed mainly by hydrolysable tannins such as punicalins, punicalagins, ellagic acid and their derivatives in agreement with the current literature [40–42]. Table 7 reports quantitative results of the main marker molecules founded in bark and peel analysed extracts for which commercial standard are available expressed as micrograms per milligrams of dried extract.

Table 7. Quantification of ellagic acid, punicalin α+β, punicalagin α, punicalagin β in bark and peel ethanolic extracts. Data represent mean values for each sample ± standard deviation (n = 2). For each compound, means followed by different lower-case letters within a row show difference between the samples ($p < 0.05$).

Compound	**Bark**		**Peel**	
	µg/mg	**RSD%**	**µg/mg**	**RSD%**
Ellagic acid	10.60 ± 0.06a	0.12	4.52 ± 0.09b	0.45
Punicalin A+B	15.80 ± 0.61a	0.78	2.51 ± 0.03b	0.24
Punicalagin A	34.90 ± 1.15a	0.67	13.24 ± 0.21b	0.33
Punicalagin B	41.16 ± 1.68a	0.82	16.27 ± 0.14b	0.17

2.3.1.2. Phytochemical analysis of leaf ethanolic extracts

In pomegranate leaf extract (Figure 2), three different chemical classes of compounds were identified: hydrolyzable tannins, flavonoids and triterpenes.

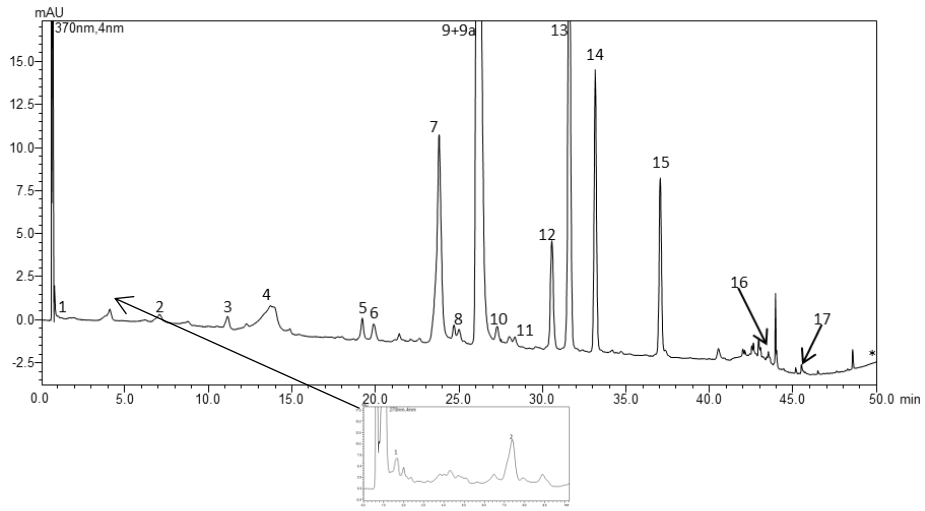

Fig. 2. LC representative chromatograms of the pomegranate leaf extract at 370 nm and the magnification at 270 nm. Compounds are numbered according to Table 8.

Table 8. List of identified and putatively identified compounds in leaf extract. Each compound is referred through its relative retention time, UV maxima λ absorption, molecular formula, pseudomolecular ions (ESI^+ and ESI^-), ion fragments generated by product ion scan mode (PIS) and identified or tentatively identified compound names. The identification confidence value and the literature reference that indicates the presence of the compounds in pomegranate are also reported.

N°	RT	λmax (nm)	Molecular formula	$[M+H]^+$	$[M-H]^-$	Supp. MW	MS^{2+} m/z	MS^{2-} m/z	Compound name	Identification confidence[b]	Ref.
1[a]	1.649	272	$C_7H_6O_5$	/	169	170		125 110	Gallic acid	1	[36]
2	7.373	314	$C_{15}H_{18}O_8$	/	325	326		163 145	Coumaric acid hexoside	2	[33]
3	11.657	273 357	$C_{13}H_8O_8$	/	291	292		247	Brevifolin – carboxyl acid	2	[23]
4	14.364	268		801 151	799	800	151	301	Ellagic derivative	2	[33]
5	19.032	270 350		611	935 655 609	610	151	301 137	Ellagitannin	3	
6	19.420	251 360	$C_{20}H_{16}O_{13}$	465	463	464	/	301	Ellagic acid glucoside	2	[39]
7	24.091	274 365	$C_{41}H_{28}O_{27}$	953	951	952	/	933 301	Galloyl-HHDP-DHHDP-hexoside (Granatin B)	2	[43]
8[a]	24.366	253 347	$C_{21}H_{20}O_{11}$	449	447	448	287	285	Luteolin 7-*O*-glucoside	1	[44]
9[a]	25.994	252 366	$C_{14}H_6O_8$		301	302	/	284 229 185	Ellagic acid	1	[23]
9a[a]	25.994		$C_{27}H_{30}O_{16}$	611	609	610			Rutin	1	[45]
10[a]	27.426	255 353	$C_{21}H_{20}O_{12}$	465	463	464	303 229 153	301 255 151	Quercetin 3-*O*-glucoside	1	[45]
11[a]	28.885	266 336	$C_{21}H_{20}O_{10}$	433	431	432	271	269	Apigenin 7-*O*-glucoside	1	[44]
12	30.971	268 332		433	431	432	271	269 195 151 117	Apigenin glycoside	3	
13[a]	31.719	267 337	$C_{21}H_{20}O_{11}$	449	447	448	287	285 257	Luteolin 4'-*O*-glucoside	1	[43]
14	33.223	268 340		449	447	448	287 153	285 151	Luteolin glycoside 1	3	
15	37.253	268 340		419	417	418	287 153	285 257 175 151	Luteolin glycoside 2	3	
16[a]	42.958	252 347	$C_{15}H_{10}O_6$	287	285	286	153 135 117	171 151 133 115	Luteolin	1	[44]
17[a]	45.538	267 336	$C_{15}H_{10}O_5$	271	269	270	163 153 119	151 117	Apigenin	1	[44]

*	50.201	-	$C_{30}H_{48}O_3$	457	455	456	333 239 191 189	407	Oleanolic acid	1	[46]
*	50.201	-	$C_{30}H_{48}O_3$	457	455	456	333 239 191 189	407	Betulinic acid	1	[46]
*	50.201	-	$C_{30}H_{48}O_3$	457	455	456	333 239 191 189	407	Ursolic acid	1	[46]

[a] Compounds identified by comparing with reference standards.

[b] An identification confidence according to the request of the CAWG (2007) is indicated: Level 1:Identified compound (A minimum of two independent orthogonal data (such as retention time and mass spectrum) compared directly to an authentic reference standard; Level 2:Putatively annotated compound (Compound identified by analysis of spectral data and similarity to bibliographic data); Level 3: Putatively characterised class compound.

Chromatographic profiles, UV and mass spectral data allowed to detect 20 informative compounds. The UV spectra detected for each peak provided a preliminary indication of the class to which the compounds belong. The molecular weight of each peak was also defined by its mass spectral pattern through the complementary correspondence between positive and negative pseudomolecular ions in ESI^+ and ESI^- modes. Product ion scan analysis of the pseudomolecular ions investigated, provided diagnostic fragments for each compound, this further structural information were also used to investigate the compounds for which pure standards are not commercially available.

The identity of 12 compounds in the extract was confirmed by co-injection of authentic commercially available standards, whereas other peaks were putatively identified by comparison their tandem mass spectrometry fragmentation pattern with those reported in literature data (Table 8). This method allowed to obtain further structural information on unknown compounds. For example, compound **7** (granatin B) was tentatively identified by its UV spectral data (UV_{max}= 274 nm), pseudomolecular ions 953 m/z and 951 m/z, in ESI^+ and ESI^- ionization modes, respectively, and fragmented to give diagnostic ions at 933 and 301 m/z in ESI^- [39]. Selected reaction monitoring acquisition (SRM) was also carried out on the specific product ions detected, giving additional structural information on the investigated compounds. Table 8 reported the main compounds identified in pomegranate leaf extract.

In agreement with current literature, flavones and flavonols represent the most representative secondary metabolites in pomegranate leaf, which often exist as glycosides of luteolin, apigenin and quercetin [47]. Phytochemical analyses revealed that ellagic acid is the most abundant compound, while hydrolyzable tannins such as punicalins and punicalagins, markers of the other parts of the pomegranate plant, as fruit, bark and peel, were not detected as supported by [41]. The following compounds represent case examples of how the fragmentation patterns were performed in order to their identification. Compounds **14** and **15** are characterized by UV maximum absorptions at 268 and 340 nm typical for A and B ring absorption, respectively in flavonoids [48]. Their fragmentation generated $[M+H]^+$ ions at 449 and 419 *m/z* and a $[M-H]^-$ ions at 447 and 417 *m/z* in positive and negative ionization mode for compound 14 and 15 respectively. The MS/MS fragmentation generated the same fragments, *m/z* 287 and *m/z* 285 in the positive and negative ionization mode respectively, due to the cleavage of glycosidic bond [49].

Moreover other diagnostic fragments such as *m/z* 153 and *m/z* 151 in the positive and negative ionization mode respectively, were generated. These data, compared to literature bibliography

enabled to identify compounds 14 and 15 as luteolin glycosides [50][44] . Compound **12** generated pseudomolecular ions at 433 in ESI^+ and 431 in ESI^- suggesting a molecular weight of 432 g/mol and showed a similar UV maximum absorption as compound **7**. MS/MS fragmentation gives a potential aglycone of 270 g/mol by a neutral loss of 162 Da both in positive and negative ionization modes. Moreover, MS/MS fragmentation in negative mode gave the same fragments as those of compound **17** making possible to consider it as a glycoside of apigenin. Likewise, luteolin and its glycosides (**8, 13, 16**) were confirmed in the extracts thanks to the co-injection of the corresponding reference standard. Ellagic acid (**9**), rutin (**9a**), apigenin and its glycoside (**11, 17**) and quercetin glycoside (**10**) were identified in the extracts with the same approach. Ellagic acid derivatives (**4, 5, 6, 7)** were tentatively identified by comparison of spectral data reported in the literature [25].

LC-MS analyses allowed also to detect the presence of $[M-H]^-$ ion at *m/z* 455 in negative ionization mode and a $[M+H]^+$ ion at *m/z* 457 with a fragment ion at m/z 439 due to a H_2O molecule loss in positive ionization mode, suggesting the presence of triterpenoid molecules [46]. Brieskorn and Fu [51,52], reported the presence of pentacyclic triterpenoid acids such as oleanolic, betulinic and ursolic acid in pomegranate bark, peel, leaf and flower. These data, together with the co-injection of the reference commercial standards, enabled to identify the presence of the triterpenoid compounds, but because of an evident coelution and a similar MS/MS fragmentation pattern in positive ionization mode 203, 189, 147, 123, 109 of the three isomeric pentacyclic triterpenoid acids, an optimization of the separation method was studied.

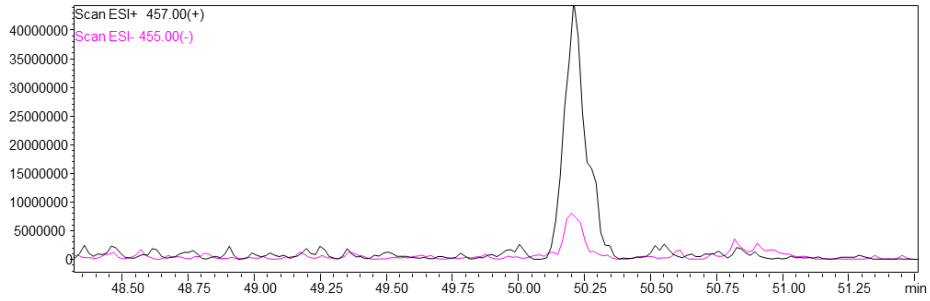

Fig. 3. MS representative chromatograms of $[M-H]^-$ ion at *m/z* 455 and a $[M+H]^+$ ion at *m/z* 457 in pomegranate leaf extract.

2.3.1.3. Method optimization and chromatographic separation of the triterpenoid acids

Recently, several methods have been evaluated for the quantitative analysis of triterpenoid acids in different raw materials. They include gas chromatography [22], thin layer chromatography [53], capillary electrophoresis [54], non-aqueous capillary electrophoresis [55], micellar electrokinetic capillary chromatography [56] and the mostly used high-performance liquid chromatography (HPLC) which has been often used for the detection of terpenoid acids because the possibility of a direct analysis and the method reproducibility [57]. For the determination of triterpenoid acids mixture, a method that involves HPLC with C18 as the solid phase [58,59] is generally used, but this often results in incomplete separation among them [60]. Wang et al. [61] developed a successfully efficient method for separation and quantitation of these triterpenes in the different Chinese herbs using gamma-cyclodextrin (γ-CD) as the mobile phase additive to improve the separation in an HPLC system. Plante [62] evaluated a C_{30} chromatographic stationary phase with a buffer solution as a mobile phase.

In a first step the separation of the triterpenoid acids in pomegranate leaf were run by testing the different HPLC column present in the laboratory such as amide, C18 and Phenyl-Hexyl with a gradient elution optimization. Since all LC analyses did not allowed to obtain a good separation, another analytical platform (GC-FID-MS) was taken into consideration, therefore the leaf extract was derivatised with BSTFA and pyridine as suggested by [16]. A hydrophobic 100% methyl polysiloxane short capillary GC column with a lenght of 15 m, narrow diameter of 0.18 mm and film thickness of 0.18 µm and able to work at temperature higher that 300°C was used. The optimized temperature program starting from 50°C (2 min) to 300°C (2 min) at 5°C/min associated to the characteristics of the column allowed to obtain an optimal chromatographic separation of the oleanolic, betulinic and ursolic acids (Figure 4).

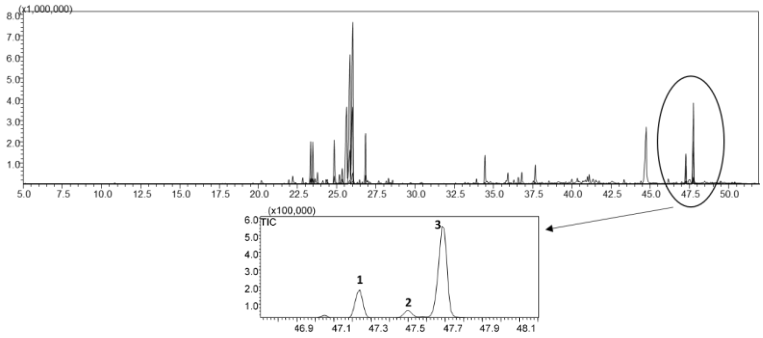

Fig. 4.
GC-MS representative chromatogram of derivatized pomegranate leaf extract with the optimal separation of the triterpenoid fraction composed by oleanolic (1), betulinic (2) and ursolic acid (3). Monosaccharide derivatives were also detected (retention time window: 22-27 minutes).

Table 9. List of identified pentacyclic triterpenoid acids found in pomegranate leaf extracts. Each compound is referred through its relative retention time, molecular formula, molecular weight, molecular weight fragments by electron impact source, and identified compound names. The identification confidence value and the literature reference are also reported.

				MS			
N°	**RT**	**Molecular formula**	**Molecular weight**	**fragmentation m/z**	**Compound name**	**Identification confidence[b]**	**Reference**
1	46.300	$C_{30}H_{48}O_3$	456	320, 203, 189, 133	Oleanolic acid	1	[16]
2	46.550	$C_{30}H_{48}O_3$	456	320, 203, 189, 133	Betulinic acid	1	[16]
3	46.750	$C_{30}H_{48}O_3$	456	320, 203, 189, 133	Ursolic acid	1	[16]

a Compounds identified by comparing with reference standards.
b An identification confidence according to the request of the CAWG (2007) is indicated: Level 1:Identified compound (A minimum of two independent orthogonal data (such as retention time and mass spectrum) compared directly to an authentic reference standard; Level 2:Putatively annotated compound (Compound identified by analysis of spectral data and similarity to bibliographic data); Level 3: Putatively characterised class compound; Level 4: unknown compound.

2.3.1.4. Phytochemical analysis using HPLC-PDA-MS/MS and GC-MS of leaf ethanolic extracts from different origin, harvesting season, year and quantitation of the main compounds

To evaluate the repeatability of the extracts, the phytochemical pattern was compared for leaves belonging to plants of different origin, harvesting season and year. Samples of pomegranate leaf were then collected in different Sardinian locations and occasionally in Piedmont, Lombardy and Greece. All dried and ground powder samples were extracted with ethanol and the extracts were

processed in SPE-C18 bond elut cartridge as described in materials and methods, to remove chlorophylls. LC gradient program optimized to 56 min in order to reduce time consuming and the same experimental conditions were applied to all samples which were analysed under the same HPLC conditions with no variation in injection volume. The identification process was similar for all samples and the quantitation of the main compounds was performed by the external standard calibration method. This method consists in a separate analysis of the reference pure standard compounds, characterized for chemical purity in the working solvent, in the same experimental conditions used for the analysis of them in the different extracts. When the reference standard is not commercially available, another compound belonging to the same chemical class and with similar behaviour towards the detector chosen for the analysis, can be used [63]. In the present study, two mixtures of standard substances were prepared. The first mixture used for LC-PDA-MS analysis was a solution of rutin, ellagic acid, luteolin 4'-*O*-glucoside, luteolin 7-*O*-glucoside, quercetin 3-*O*-glucoside, apigenin 7-*O*-glucoside in 95:05 acetonitrile/water.

The second mixture for GC-FID-MS analysis consisted of ursolic acid, betulinic acid and oleanolic acid initially dissolved in acetone, then evaporated and derivatized with BSTFA and pyridine. The standard mixtures were prepared and serial dilutions were injected to build a multi-point calibration curve. A calibration curve is a graphical representation of the quantity and response data for a single analysed compound obtained from several concentrations of it. The peak area is plotted on the y axis while the standard concentration is plotted on the x axis, they represent the dependent and the independent variable, respectively. An equation describing the best line through the data points is derived from a regression analysis of the calibration curve.
The R^2 coefficient, which ranges from 0 to 1, represent how data points is fitting in the straight line. Coefficient value closer to one represent a better curve linearity [64]. As reported in Materials and methods, the detector response for each calibration curve was linear (R^2> 0.997).

The calibration mixtures were analysed in duplicate for each concentration. Ellagic acid and luteolin 4'-*O*-glucoside were quantified with PDA detector by area measurements at their wavelength of maximum absorbance (λ max) and they were used to quantify further compounds belonging to the same chemical class, while luteolin 7-*O*-glucoside, rutin, quercetin 3-*O*-glucoside, apigenin 7-*O*-glucoside, apigenin and luteolin were quantified with selected reaction monitoring (SRM) mode because of their coelution. Triterpenoid acids were quantified through SIM mode in GC-MS system. Analytical repeatability was examined by determining the %RSD of the areas integrated at the λ max of the compounds between two analyses of the same extract. The %RSD were lower than ten for standard mixtures analysed in LC-MS and for derivatised standards mix analysed in GC-MS. Extraction procedure repeatability was checked by determining the %RSD between the averages of the areas obtained from the two extraction replicates at the λ max of quantified compounds with %RSD less than fifteen.

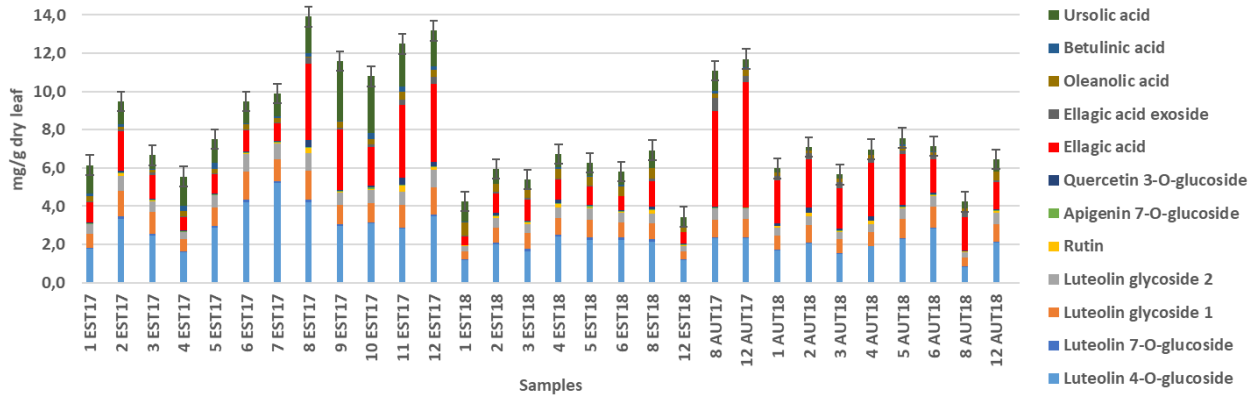

Fig. 5. Histograms representative quantification of the main components identified in pomegranate leaf extracts belonging to samples collected in different locations, season and year. Quantified compound concentration is expressed as milligram per gram of dry matrix.

Figure 5 reports the quantitative results on pomegranate leaf extracts and highlights the quantity of each target compound in relation to the total amount of quantified compounds. Apigenin and luteolin aglycon were found only in traces (< 0.05 µg/mL) while luteolin 4'-*O*-glucoside and ellagic acid are two of the most abundant compound in the leaf samples [41,65,66]. The latter was found more abundant in samples collected in October, that is coeherent with the current literature. Xiang [67] showed the ellagic acid content in leaf was the highest in September and October for most pomegranate varieties they analysed. Lingling Han [68] by analysing the composition and the contents of polyphenols in pomegranate fruit at different stages, demonstrated that the ellagic acid concentration slowly decreased with the increase of the fruit weight during fruit ripening.

It has been found that in early spring the photosynthesis is enhanced and primary metabolism is upregulated to construct leaf morphology but in the meanwhile the synthesis of total phenolics and flavonoids declines and their contents constantly decreases. Increases of the total phenolics and flavonoids in pomegranate leaf resume after May, presumably once the growing period is over [31]. Factors which may affect the differences on occurrence of secondary metabolites in pomegranate leaf can include the limitation of sample size, the different geological regions and climate changes [69].

The results of the quantitative analysis revealed some differences among the main classes of components, then a statistical analysis to highlight the significativity of this variability was performed.

2.3.2. PCA of the identified compounds of the leaf extracts

Principal Component Analysis (PCA) was adopted to highlight similarities and differences among the samples and to give a simple visualization of the main relationships between the samples on the basis of the quantified main compounds as reported by [50]. PCA is an unsupervised multivariate data analysis technique that reduce the multivariate space where objects are distributed allowing to visualize similarities and/or differences into multivariate data of secondary metabolite composition [70]. The dataset of the 30 leaf samples investigated was thus submitted to PCA by using a targeted approach based on the 12 previously characterized and quantified compounds listed in Figure 5.

Score and loading plots were built with the aim to verify if the differences on the chemical composition related to the discriminating variables had a significantly statistical discrimination. Figure 6 reports the first component (PC1) that explains 39.14% of the variation and the second component (PC2) explaining 19.72% of the variation. The influence of the variables in the distribution of the samples is shown in the related loading plot (Figure 7), where most of the compounds are negatively correlated with the first principal component. These results indicated a homogenous distribution of the samples. The elaboration showed not significant statistically differences on phenolics and triterpenes quantified among all the samples and it can be ascribed to the normal phenotypical variability and the influence of environmental factors, such as soil composition, temperature, rainfall and ultraviolet radiation which can affect the concentrations of these compounds [71,72].

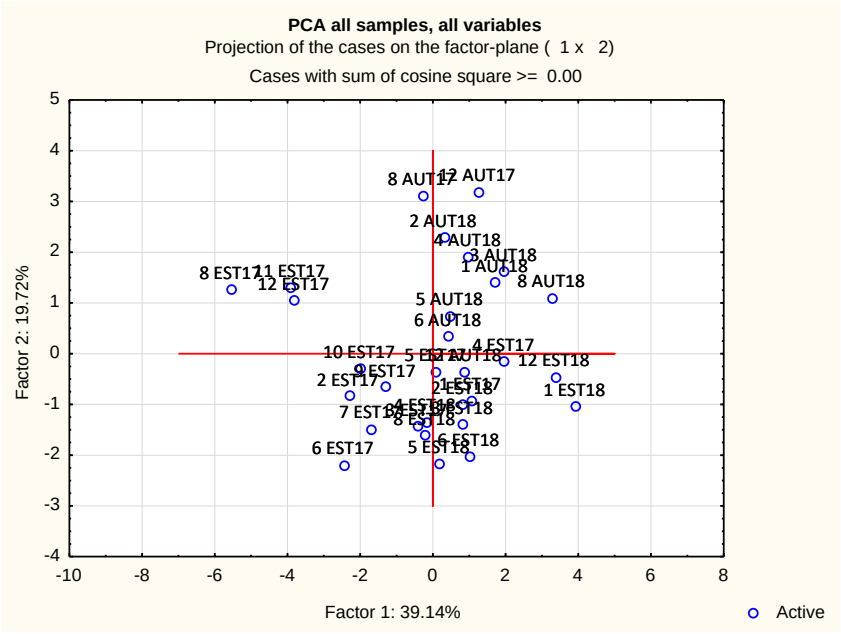

Fig. 6. Pomegranate leaf score plot of PC1 against PC2 which all samples analysed are included.

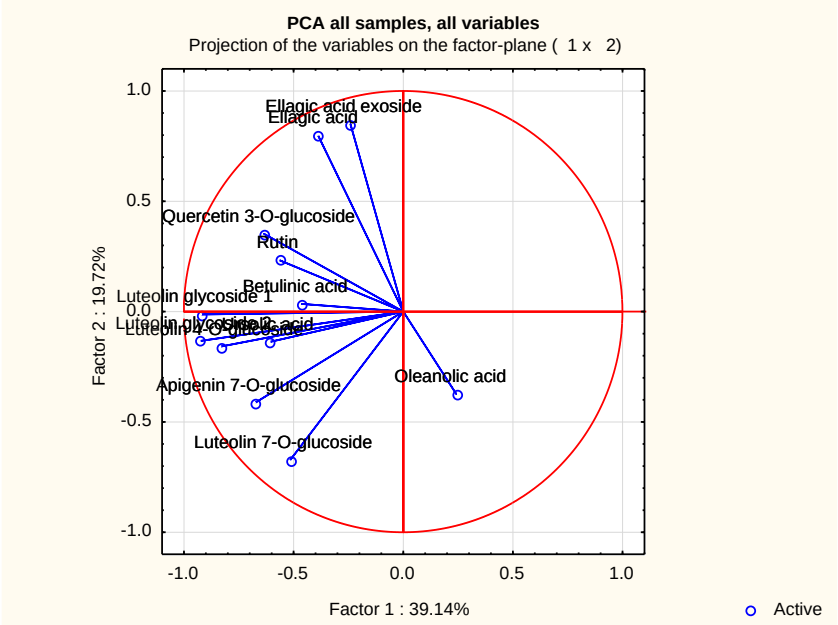

Fig. 7. Pomegranate leaf loading plot of PC1 against PC2 obtained from quantified compounds.

2.3.3. Sequence analysis of pomegranate samples from eleven different sites

To discriminate and identify plant samples, different PCR-based methods can be used, among them DNA barcoding which consists in the amplification and sequencing of specific DNA regions. The nuclear internal transcribed spacer region (*ITS)* gene and the chloroplast photosystem II protein D1 (*psbA-trnH)* gene are often used for the discrimination at the species level. *ITS* region comprises the internal transcribed spacers 1 and 2, the 5.8S ribosomal RNA gene and the partial sequences of the 18S and 28S ribosomal RNA genes [11–13], while *psbA-trnH* represents the noncoding intergenic spacer of the photosystem II protein D1 [50,73,74]. The genetic variability of pomegranate cultivars or populations has been the focus of different works, with particular attention on samples originating from Iran, Morocco, Tunisia and India. Several PCR-based methods were employed on this purpose, e.g. inter simple sequence repeat (ISSR) markers, microsatellite DNA markers (SSR), random Amplification of Polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), directed amplification of minisatellite DNA (DAMD), sequence-related amplified polymorphism (SRAP), DNA barcoding [15,75–80].

This work is focused on DNA barcoding since few literature data reports the sequencing and analysis of these genes from *P. granatum* [15,80] . A study that evaluated the genetic variability of pomegranate leaf samples from different Iranian locations shows a genetic diversity of 2.91 % for *psbA-trnH* and 0.44 % for *ITS* region, providing that *psbA-trnH* region has the highest intra-species variation and is more suitable for determining intra-species relationships of pomegranate [15].

In this work, one nuclear region (*ITS*) and one chloroplast gene (*psbA-trnH*) were amplified and sequenced for each site where pomegranate leaf samples were harvested. The *ITS* and *psbA-trnH* barcoding regions present in GenBank database originate mainly from Iran, Tunisia, China, India and only few sequences are from Italy. The aim of this work is to add additional data on *Punica granatum* from Italy and Greece to better evaluate its genetic stability or variability. Sequences obtained in this work were deposited in GenBank (Table 10) and are approximately 681 bp for *ITS* and 426 bp for *psbA-trnH*.

Table 10. List of the sequences obtained from *P.granatum* samples deposited in GenBank.

Sample	*GenBank Code for ITS*	*GenBank Code for psbA-trnH*
PGF1	MT007587	MT023064
PGF2	MT007588	MT023065
PGF3	MT007589	MT023066
PGF4	MT007590	MT023067
PGF5	MT007591	MT023068
PGF6	MT007592	MT023069
PGF7	MT007593	MT023070
PGF8	MT007594	MT023071
PGF10	MT007595	MT023072
PGF11	MT007596	MT023073
PGF12	MT007597	MT023074

In Figure 8 and 9, the comparison of *ITS* and *psbA-trnH* nucleotide composition among the eleven sites is shown. Both regions show no nucleotide variation among the samples, suggesting a stability of these biomolecular markers for *P. granatum*. Only one ambiguous nucleotide is present at

position 466 of the *ITS* region, but this could be to an inaccuracy in the sequencing. A consensus sequence was obtained from the alignment of all the samples for each region. The comparison of the *ITS* and *psbA-trnH* consensus with all the sequences present in the database, through the blast tool, revealed the presence of fifty-nine *P. granatum ITS* sequences originating from India, Iran and China and twenty-seven *P. granatum psbA-trnH* sequences from Iran, Tunisia, China and Italy (Apulia, Latium, Sardinia, Padoa and Trieste).

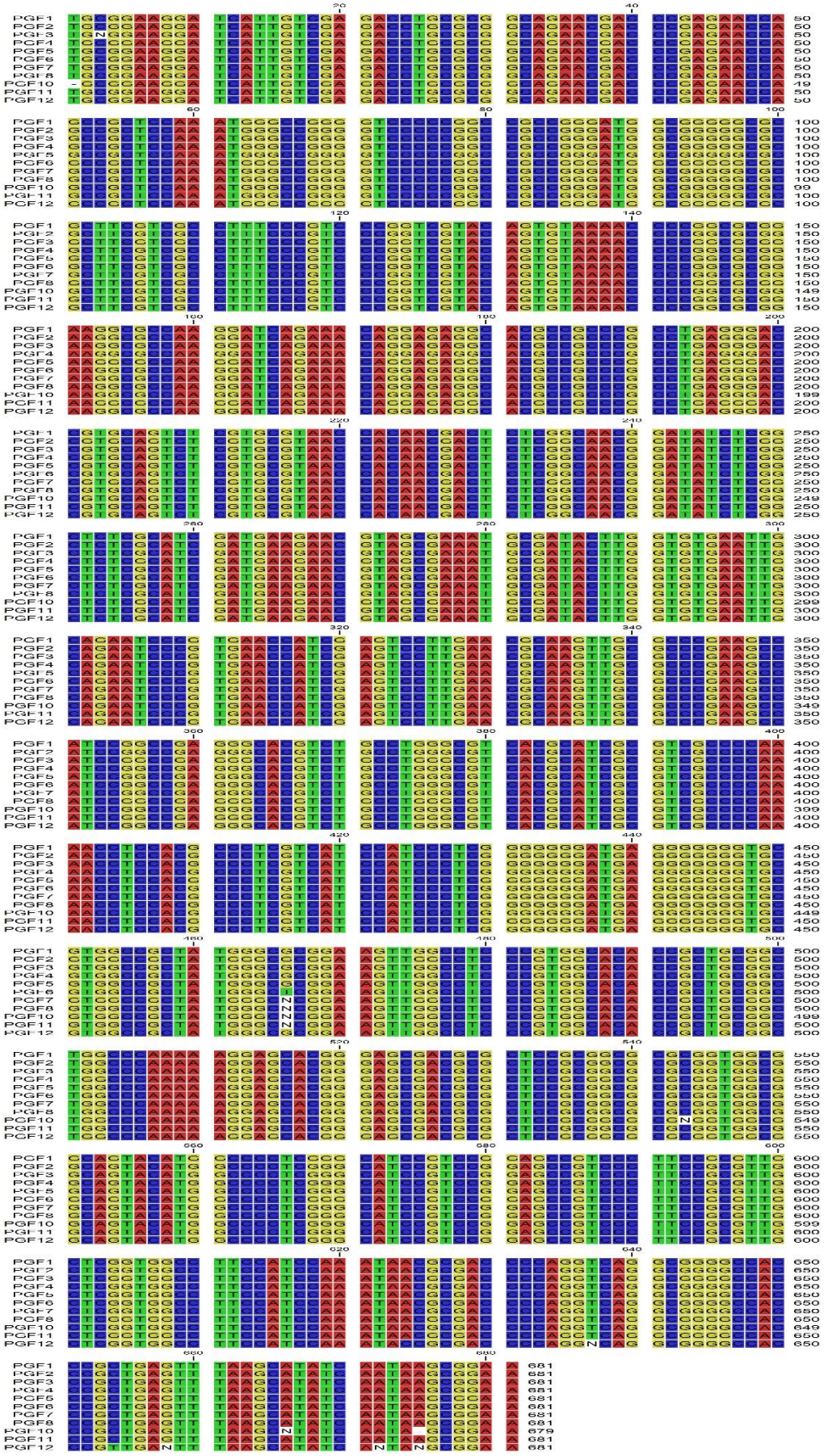

Fig. 8. Comparison of *ITS* sequences between the eleven *Punica granatum* samples; Red: Adenine; Blue: Cytosine; Yellow: Guanine; Green: Thymine; N: any base.

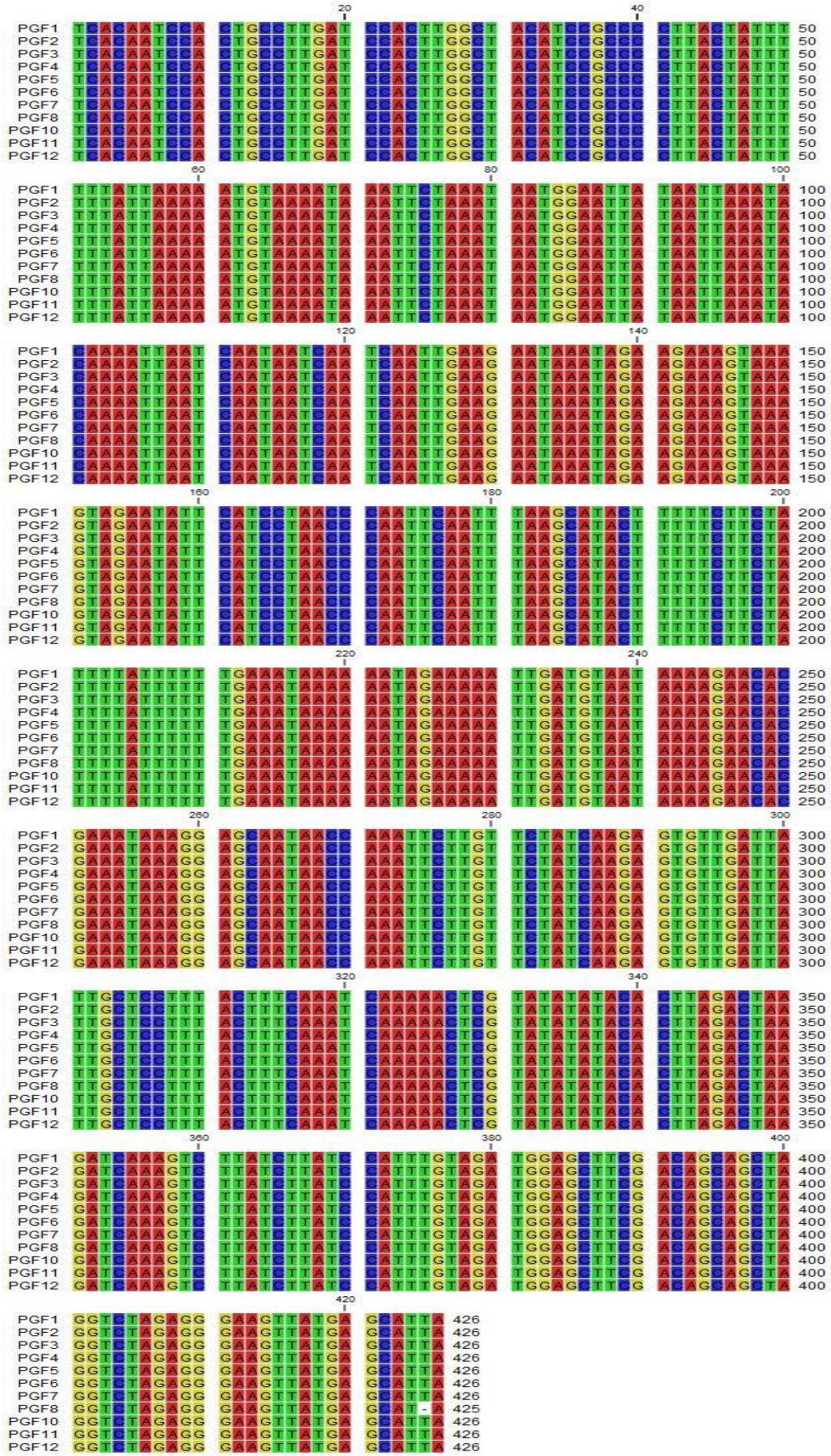

Fig. 9. Comparison of *psbA-trnH* sequences between the eleven *Punica granatum* samples; Red: Adenine; Blue: Cytosine; Yellow: Guanine; Green: Thymine; N: any base.

Concerning *ITS* region, a percent of identity ranging from 100% to 96.89% was observed by comparing the consensus with the database. Indian Arakta cultivar (JQ655166.1), was the sample with the highest nucleotide variability (about 20 different nucleotides). The GenBank comparison with the other species belonging to the same genus, namely *Punica protopunica,* an endemic tree of the island of Socotra, revealed a percentage of identity of 91.15% (FM887009.1). These data confirms an higher inter-species variability and an intra-species stability. Even if pomegranate sample present in database are from different sites all over the world the lowest percentage of identity among them is still satisfactory compared to those obtained comparing *P. granatum* and *P. protopunica*. These results confirm the use of *ITS* region to carry out a proper discrimination of the species. The *psbA-trnH* comparison with the GenBank data on *P. granatum* showed a percent identity values from 100% to 97.64% and it is interesting to notice that the Italian samples deposited shows 100% similarity, supporting the stability of the species in Italy. In addition, literature reports that no intraspecific sequence variation was found at *psbA-trnH* level in *P. granatum* sampled in the wild and in botanical gardens in Italy, confirming the stability of *psbA-trnH* in this area [14]. Conversely no data about this gene is available for *Punica protopunica* species but alignment results among *P.granatum psbA-trnH* sequences are consistent with those obtained for the *ITS* gene. Despite literature data showed that *psbA-trnH* region is more variable compared to *ITS*, in our study both regions showed stability among the samples analysed.

In conclusion, this study contributes to increase the current data about pomegranate genotype for *ITS* and *trnH-psbA* genes. Concerning *ITS* gene, this is the first time that Italian samples have been sequenced, providing information about a new geographical origin. On the other hand, the data on *psbA-trnH* implement the current knowledge present in GenBank, providing sequences from different locations such as Sardinia, Piedmont and Greece, and confirming the stability of this molecular marker from five Italian regions [14].
Future studies should be focused on the sequencing of more DNA regions for *Punica granatum* species sampled in different geographical origin in order to add information to the current literature, obtaining a worldwide overview of the variability / stability of the species. Further genotypic information about the other species of the genus: *P. protopunica,* are necessary to build a solid database with more comparable data. These information will help for the use, management and conservation of genetic resources for breeding and selection programs [76].

2.4. Conclusions

Plants contain most of the active molecules found in traditional medicine and their extracts have been studied for long time in pharmaceutical and academic institutes. However, there is still much that deserve to be discovered and studied [75]. *Punica granatum* (pomegranate), an ancient and beloved fruit, is known to have many healthy properties used in the past in traditional medicine [1]. Today it is cultivated in several regions around the world and its fruit is well consumed as such or as juice. Several studies correlated its beneficial properties to specialized biosynthesized metabolites, especially polyphenols [76]. This study started in collaboration with the Department of Life and Environmental Sciences of the University of Cagliari and reports the phytochemical investigation of non-edible parts of pomegranate. The attention was focused on bark, peel and leaf ethanolic extracts potentially active on the *in vitro* inhibition of two key functional HIV-1 enzymes: reverse transcriptase-associated RNase H activity and integrase.

The LC analysis of polyphenols with an RP-Amide column gave enough resolution, enabling the chromatographic separation of all the main target compounds. These compounds were identified and quantified through HPLC-PDA-ESI-MS/MS. From the results, supported by literature data, it can be assumed that pomegranate bark and peel contain ellagitannins as main polyphenols and punicalins, punicalagins and ellagic acid represent marker compounds in these matrices [42,77]. In this study, the attention was more focused on the little studied pomegranate leaf, in comparison with the literature data of the other edible and non-edible parts of pomegranate [3,6,23,31,65]; moreover, leaves' collection is a sustainable approach as it doesn't cause damage to the plant during the pruning. A particular combination of techniques, as HPLC-PDA-ESI-MS/MS and GC-MS allowed to detect besides phenolic acids, ellagitannins and flavonoids also an interesting triterpenoid fraction composed by oleanolic, betulinic and ursolic acid [16].

To verify the possible variability of the leaf extract, leaf samples were collected from different sites in Sardinia and occasionally from other regions, in 2017 and 2018, in summer and autumn season. The extraction method was optimized in reproducibility and exhaustivity. The phytochemical pattern of the different samples showed an equivalent qualitative composition with some quantitative differences in particular for ellagic acid: its amount in leaves is increasing after summer while in fruits is decreasing during ripening as reported in literature [67,68]. Principal component analysis showed not significant statistically differences among the samples. Differences are then to be attributed to the normal phenotypical diversity due to environmental factors [69]. It has been observed the noticeable presence of large hydrolysable tannins in fruit peel and bark while in leaf are in a little amount respect of the flavonoid fraction composed by luteolin, apigenin and quercetin glycosides. Hydrolysable tannins included gallotannins or ellagitannins which are polyesters of organic acids: gallic and ellagic acids respectively [78]. Their formation might explain a lower concentration of ellagic acid in bark and peel compared to that detected in the leaf extracts.

Moreover, biomolecular analysis was performed by sequencing two barcoding genes: *ITS* and *psbA-trnH*. Results showed no differences in the nucleotide composition among all the investigated pomegranate samples from Sardinia and other locations. The outcome obtained from the combination of chemical and molecular techniques allowed to attribute the similarity of the samples under a phytochemical and biomolecular point of view.

In conclusion, these results can be of great scientific interest because they represent detailed phytochemical data on the non-volatile fraction of pomegranate leaf considering several individuals and harvesting periods. In addition, they are intended to supplement the current literature on pomegranate, demonstrating that also non-edible parts of the plant are a promising source of bioactive compounds of pharmaceutical, cosmetical and food fields of interest, and deserve to be deeply studied.

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Chapter 3

Bioguided fractionation and biological activity of pomegranate leaf extract on viral targets and multiple myeloma cells

CHAPTER 3: BIOGUIDED FRACTIONATION AND BIOLOGICAL ACTIVITY OF POMEGRANATE LEAF EXTRACT ON VIRAL TARGETS AND MULTIPLE MYELOMA CELLS

3.1. Introduction

Current literature data attributes long history and many healthy properties on pomegranate including antibacterial antiviral and anticancer properties [1–6]. In addition, studies on its edible and non-edible parts show *in vitro* induction of apoptosis on multiple myeloma cells [7,8].

In this PhD project, the potential antiviral activity of pomegranate leaf extract against the human immunodeficiency virus 1 (HIV-1) (a) and Zika Virus (ZIKV) (b) were studied.

a) AIDS is classified as an epidemic disease caused by the infection of HIV. It is a relevant problem that nowadays affects millions of people around the world. The use of antiretroviral combination drugs represent an effective therapy for delaying progression to AIDS and to allow the immune system of HIV-infected individuals to be reconstituted [9]. The developed resistance to the most of commercially available drugs by the virus, justify the development of new targets. Studies identified three major enzymes regarding the viral replication cycle: HIV-1 protease (PR), HIV-1 reverse transcriptase (RT) and HIV-1 integrase (IN) [10]. The latter allows the integration of the viral genome into the host chromosome, a fundamental step in the viral replication process. HIV-1 IN has recently obtained a particular interest because a similar enzyme in not present in the human body. For this reason, the research is addressed in the development of novel drugs by targeting different sites of this enzyme [11]. To date, in order to make difficult for the virus to develop resistance, the most promising class of HIV-1 inhibitors is represented by the dual HIV-1 IN and RT inhibitors targeting for their capacity to interact with two different target enzymes [12]. In this context, literature data attributes to the juice from pomegranate fruit an AAAA microbicide candidate; accessible, acceptable, affordable and accelerative availability to marketing [13].
Following the promising preliminary results obtained with the crude pomegranate leaf extract from the botanical garden of Cagliari which highlighted a possible inhibition of both HIV-1 IN and RT, and since literature reports the *in vitro* capability of ellagic acid, the major marker of the extract, to inhibit these enzymatic activities [14,15], a bio-guided fractionation of the extract was performed on silica column chromatography in order to select new bioactive molecules from the flavonoid enriched fractions, tested on the viral enzymatic target.

b) Zika virus infection represents the cause of a recent global health crisis subsequently its entrance into Latin American population and this induced to the declaration of a Public Health Emergency of International Concern (PHEIC) by the WHO in early 2016 [16,17]. Zika virus (ZIKV) is classified as an enveloped positive strand RNA virus and it is a member of the Flaviviridae family, mostly transmitted by *Aedes aegypti* mosquitos [18]. ZIKV represents an emerging infectious viral pathogen; while the disease is not associated with high mortality rates and the clinical manifestations of the infection cases are not serious, it can carry out a severe impact on fetal development leading to cerebral anomalies namely microcephaly and the paralytic Guillain-Barrè syndrome in adults [17]. No vaccine or specific antiviral agent against ZIKV is available nowadays [19]. Ellagic acid showed *in vitro* antiviral activity such as mentioned for HIV-1 [14,20], HPV [21], HSV-1 [22], hepatitis B [23] and C virus [24], human influenza virus A [25], and Rhinoviruses [26] and it is also active on Dengue virus

[27] which belongs to the same genus of Zika virus. A recent study showed that the Polyphenol-Rich Extract from *Psiloxylon mauritianum* inhibits the infection of ZIKV *in vitro* without exhibiting cytotoxic effects [27].

As a rich source of polyphenols, a bio guided fractionation using chromatographic techniques on pomegranate leaf ethanolic extracts was performed. Biological assays were carried out against the two strains of the ZIKV: 1947 Uganda (MR766), considered more virulent, and the Asian-lineage (HPF2013) [19]. Beside the fractionation of the phenolic and triterpenoid enriched fraction from the total extract, ellagic acid was also purified using a preparative LC. In addition, to deeply investigate the mechanism of action of the ellagic acid, virus inactivation and time of addiction assays were performed.

Considering literature data for the potential anticancer properties of some classes of specialized metabolites as polyphenols and terpenoids [28–30], during the PhD project , the anticancer potential of pomegranate leaf extracts, fractions and pure compounds were tested on acute lymphoblastic leukaemia (ALL) and multiple myeloma (MM) cell lines in collaboration with the Pharmaceutical biology group (Prof. Thomas Efferth) of the Johannes Gutenberg University of Mainz. Hematopoietic cancers (HCs) are described as malignancies of immune system cells. Leukaemia and multiple myeloma are the most common hematologic malignancy characterized by the clonal expansion of white cells [31]. Due to the advances in therapy, and of novel molecular targets (immunomodulatory drugs, monoclonal antibodies and proteasome inhibitors), a great improvement in patients' survival over the past decade has been observed. Nowadays, the treatment of refractory and relapsed disease remains the main challenge which provides to overcome the development of drug-resistance in the clinical oncology by seeking and developing new cytotoxic agents.

Multidrug resistance (MDR) is considered an ability of cancer cells, acquired or inherited, to become resistance to different chemotherapeutic agents which target different cellular molecules [32,33]. Cellular or genetic alteration acquired during the development of drug resistance, involves the overexpression of the ATP-binding cassette (ABC) transporter which increases the efflux of cytotoxic drugs. P-glycoprotein (P-gp, ABCB1/MDR1) is the most studied transporter of this family, it is overexpressed in many cancer types and is responsible for drug efflux and resistance to several drugs [34]. Many studies have documented the effectiveness of botanicals and phytochemicals to fight cancer MDR [35].
On the basis of the literature bibliography dealing with the proapoptotic properties of pomegranate juice on different leukaemia cell lines [36] a preliminary citotoxicity assay on leaf extracts, SPE fractions and pure marker molecules was performed on Human T lymphoblast obtained from peripheral blood of a child with ALL (CCRF-CEM) and its multidrug-resistant P-glycoprotein-overexpressing subline CEM/ADR5000 cells [37]. Then, considering the current literature on the cytotoxicity of ursolic acid against *in vitro* MM cells [38], the pentacyclic triterpenoid acid detected in pomegranate leaves [39] was tested on nine MM cell lines and on human peripheral mononuclear cells (PMNC). Finally, one of the most responsive MM cell line, RPMI8226 was selected for cytofluorimetric analyses to detect the cell cycle distribution, apoptosis induction and reactive oxygen species as performed by Mbaveng et al. [40].

3.2. Materials and methods

3.2.1. Bioguided study on HIV-1 antiviral activity

3.2.1.1. Plant materials and sample preparation

The ethanolic extract of pomegranate leaves was provided by the Department of Life and Environmental Sciences of the University of Cagliari. Leaves were collected in the Botanical garden of Cagliari and a voucher specimen (CAG 514/A) deposited at the same University.

3.2.1.2. Chemicals

HPLC-grade acetonitrile (LC-MS grade), petroleum ether, ethyl acetate, silica gel (SIGMA-ALDRICH® pore size 40 A, 70-230 mesh particle size) methanol, formic acid (>98% purity), ellagic acid, apigenin, were purchased from Merck (Milan, Italy). De-ionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Luteolin, apigenin 7-*O*-glucoside, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 4'-*O*-glucoside, were purchased from Extrasynthese (Genay Cedex, France).

3.2.1.3. Column chromatography fractionation

Stationary phase: Silica gel (20 g). Mobile phase: increasing polarity gradient from petroleum ether (Pe) to ethyl acetate (EtOAc) and methanol (MeOH). Loading: pomegranate leaves crude extract (1 g) adsorbed on silica gel (3 g). Fraction volume: 20 mL. The elution was monitored with TLC on 20 × 20 cm silica gel 60 aluminum plates coated with fluorescent indicator F_{254} (Merk, Germany) by using different solvent mixtures composed by Pe/EtOAc and EtOAc/MeOH in a glass chamber. In addition, the solvent system composed by toluene, ethyl acetate and formic acid (6:4:0.8 v/v/v), was used to obtain a better run and separation of ellagic acid from other components [41]. The detection of spots was obtained under UV light at 254 nm and subsequently through carbonization with 5% (v/v) H_2SO_4 methanolic solution.

3.2.1.4. HPLC-PDA-MS/MS analysis

For the obtained fractions, a 10 mg/mL stock solution in methanol was prepared, subsequently diluted to 5 mg/mL with acetonitrile/water (95:5, v/v) and filtered through a 13 mm diameter, 0.22 µm PTFE syringe hydrophilic filter and injected in the HPLC-PDA-MS/MS system and the main components were quantified as mentioned in chapter 2.2.4. and the results expressed as µg of compound per mg of dry fraction (µg/mg).

3.2.1.5. HIV-1 RT RNase-H and IN inhibition assays

HIV-1 RT RNase-H assay: The HIV-1 RT-associated RNase H activity was calculate in 100 μl reaction volume containing Tris–HCl (50 mM), pH 7.8; $MgCl_2$ (6 mM); dithiothreitol (DTT) (1mM); KCl (80 mM); hybrid RNA/DNA (5'-GTTTTCTTTTCCCCCCTGAC-3'-fluorescein), 5'-CAAAAGAAAAGGGGGGACUG-3'-Dabcyl); and of RT (3.8 nM). After the incubation time of 1 h at 37 °C, by EDTA addition the reaction was stopped and products were measured with a Victor 3 (Perkin) plate reader at 490/528 nm. Homogeneous Time-Resolved Fluorescence (HTRF) LEDGF-dependent assay: The IN LEDGF/p75 dependent assay allowed to calculate the inhibition of the 3'-processing and strand-transfer IN reactions in the presence of recombinant LEDGF/p75 protein. Shortly, IN (50 nM) was preincubated with increasing concentrations of total pomegranate leaf extract, fractions and compounds for 1 h at room temperature in reaction buffer containing HEPES (20 mM, pH 7.5), DTT (1 mM), glycerol (1 %), $MgCl_2$ (20 mM), Brij-35 (0.05 %), and BSA (0.1 $mgmL^{-1}$). To this mixture, DNA donor substrate, DNA acceptor substrate, and LEDGF/p75 protein (50 nM) were added, and incubation was performed at 37 °C for 90 min. After the incubation, europium/streptavidin (4 nM) was added to the reaction mixture and the HTRF signal was recorded with a PerkinElmer Victor 3 plate reader and used at λ_{ex}=314 nm and λ_{em}=668 and 620 nm for the acceptor and donor substrates, respectively as described by Esposito et al. [42].

3.2.2. Bioguided study on Zika antiviral activity

3.2.2.1. Plant materials and sample preparation

The leaves belonging to the different samples collected in summer season were mixed to obtain an homogeneous sample to prepare the summer extract while the leaves collected in autumn in the botanical garden of Cagliari were extracted to prepare a sample representative of autumn extract, because at that time other samples collected in autumn season were not available. The extraction method was the same already described in chapter 2.2.3.

3.2.2.2. Chemicals

HPLC-grade acetonitrile (LC-MS grade), petroleum ether, ethyl acetate, silica gel (SIGMA-ALDRICH® pore size 40 A, 70-230 mesh particle size) methanol, Pyridine, BSTFA, formic acid (>98% purity), ellagic acid, rutin, apigenin, were purchased from Merck (Milan, Italy). De-ionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Luteolin, apigenin 7-*O*-glucoside, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 4'-*O*-glucoside, betulinic acid, oleanolic acid and ursolic acid were purchased from Extrasynthese (Genay Cedex, France).

3.2.2.3. SPE-C18 cartridge and LC-PREP fractionation

Crude ethanolic extracts were fractionated with SPE-C18 cartridge: 30 mg of each crude extract were resuspended in 1 mL of methanol/water (20:80, v/v), loaded on Bond Elut Jr 500 mg SPE-C18

cartridge (Agilent Technologies, USA), first eluted with 5 mL of methanol/water 85:15 v/v (PG85 A), subsequently eluted with 5 mL of methanol/water 95:05 v/v (PG95) and evaporated in a rotary evaporator. This operation was repeated several times to obtain a sufficient amount of each fractions for both extracts. Fraction PG85 characterized by the presence of the phenolic compounds, was then further fractionated in a LC system; the fraction at the concentration of 40 mg/mL was injected in a SHIMADZU LC-10AT system using an Ascentis Express RP-Amide column (15cm x 10mm, 5 μm, Supelco, Bellefonte, USA) with water/formic acid (999:1, v/v) and acetonitrile/formic acid (999:1, v/v) as mobile phases A and B, respectively. The flow rate was 1 mL/min and the column temperature was maintained at 30°C. The gradient program was as follows: 10% B for 1 min, 10–30% B in 60 min, 30–51% B in 9 min, 51–100% B in 1 min, 100% B for 4 min. Total pre-running and post-running time was 82 min. Injection volume: 100 μL. In these conditions ellagic acid peak was collected several times, the organic solvent evaporated with rotary evaporator and the sample was subsequently freeze-dried. Fraction PG95 was characterized by the presence of the triterpenoid acids. SPE fractions A and B were dissolved into acetonitrile/water (95:5, v/v) at 2.5 mg/mL and filtered through a 13 mm diameter, 0.22 μm PTFE syringe hydrophilic filter and injected in the HPLC-PDA-MS/MS system and GC-MS system and the main components were quantified as mentioned in chapters 2.2.4 and 2.2.5.

3.2.2.4. Isolated ellagic acid characterization: HPLC-PDA-MS and ^{1}H NMR

Isolated ellagic acid dissolved in methanol at the concentration of 1 mg/ml, was injected for LC-MS analysis as mentioned in chapter 2.2.4. The peak percentage area in the chromatogram was obtained at the wavelength of 254 nm.
Isolated and commercial ellagic acid (1.5 mg) were dissolved in 700 μL of DMSO-d6, and NMR spectra (600 MHz for ^{1}H) were recorded. Chemical shifts were calibrated to the residual proton resonances of the solvent: DMSO-d6 (δH = 2.54, δC = 39.5). Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz.

3.2.2.5. Cell culture

African green monkey fibroblastoid kidney cells (Vero, ATCC CCL-81) were grown as monolayers in Eagle's Minimum Essential Medium (MEM) (Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic solution (Penicillin-Streptomycin, Sigma-Aldrich) in humified 5% CO_2 atmosphere at 37° C. The antiviral assays against ZIKV were performed on Vero cells using MEM medium supplemented with 2% FBS.

3.2.2.6. Virus

Two ZIKV strains were used to investigate the antiviral potential of pomegranate: the 1947 Uganda MR766 and the 2013 French Polynesia HPF2013, representing the African and the Asian lineage respectively. The viruses were produced by transfection of 293T cells, as described in [19]. Ugandan ZIKV-MR766 laboratory strain and ZIKV-HPF2013 were propagated and titred in Vero cells. Titration was performed in 96-well plates infecting pre-seeded Vero cells (6 X 10^3/well) with serial dilutions of both viruses; following 2 hour-infection, the inoculum was removed, and cells overlaid with 1.2 % methylcellulose. After 72-hour-incubation at 37°C 5% CO_2 atmosphere, the cell monolayers were fixed, stained with 0.1% crystal violet in 20% ethanol, and the viral plaques were counted. The mean

number of plaques was multiplied by a dilution factor to obtain the titer and expressed as plaque-forming units (PFU) per milliliter.

3.2.2.7. Viability assay

Cell viability was assessed using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] MTS assay as described in [43]. The effects of summer and autumn pomegranate leaf extracts, fractions, and ellagic acid on Vero cells viability were evaluated at 24 and 72 hours. Vero cells were seeded at a density of 10×10^3/well and 6×10^3/well in 96-well plates for 24 and 72-hour assays, respectively. The following day, they were incubated with serial dilutions of pomegranate extracts, fractions or ellagic acid in duplicate for 24 and 72-hours. Cell viability was determined using the Cell Titer 96 Proliferation Assay Kit (Promega). Briefly, at the end of the incubation period, following a wash with MEM, 20 µL of MTS reagent was added to 100 µL MEM per well, and incubated at 37 °C for 4 hours. MTS is bioreduced by metabolically active cells into a colored formazan product. Absorbances were measured at 491 nm using Multiskan™ FC Microplate Photometer (Thermo Scientific). The effect of pomegranate extracts and fractions on cell viability at different concentrations was expressed as a percentage of absorbance values of treated cells compared with those of cells incubated with culture medium alone. The 50%-cytotoxic concentrations (CC_{50}) and 95% confidence intervals (C.I.) were determined with Prism 5 software (Graph-Pad Software).

3.2.2.8. Virus plaque reduction assay

The antiviral activity of pomegranate leaf extracts, fractions and ellagic acid was determined by viral plaque reduction assay on Vero cells. Vero cells were seeded in 24-multiwell plates at $6 \text{ X } 10^4$/well density in MEM 10% FBS. The following day, the cells were treated with serial dilutions of the pomegranate extracts, fractions, or pure compounds for 2 hours in MEM-2% FBS. After this step, the cells were infected with ZIKV-MR766 or ZIKV-HPF2013 (multiplicity of infection, MOI PFU/cell 0.005) in presence of serial dilutions of the investigated extracts, fractions and compounds, for 2 hours at 37°C. After a wash with medium, the cell monolayers were incubated with 1.2%-methylcellulose MEM medium 2% FBS containing serial dilutions of the investigated substances for 72 hours. After fixing and staying with 0.1% crystal violet in 20% ethanol, the ZIKV plaques were microscopically counted.

Results are reported as percentages of viral plaques in comparison to controls for each concentration of the investigated substance. The concentration of extracts, fractions, and ellagic acid that reduced viral infectivity by 50% (half maximal effective concentration, EC_{50}) and concentrations that reduced viral infectivity by 90% (effective concentration-90, EC_{90}) were calculated by the program Prism (variable slope-sigmoidal dose–response curve). The results are reported for three experiments performed in duplicate. The Selectivity Index (SI) was calculated as the ratio CC_{50} / EC_{50}.

3.2.2.9. *Virus inactivation assay*

Ellagic acid was investigated for its ability to directly inactivate ZIKV particles. Ellagic acid dilutions was incubated with ZIKV-MR766 (1 X 10^5 PFU) at concentrations corresponding to EC_{90} values (µg/mL) as obtained in the viral plaque reduction assay. The mixtures were incubated for 2 h at 4°C and 37 °C. Dilutions from both treated and untreated ZIKV-MR766 aliquots were titrated to non-inhibitory concentrations on Vero cells. After 72-hour incubation at 37°C, the cells were fixed and stained with 0.1% crystal violet in 20% ethanol, and the viral plaques were counted. Viral infectivity was determined microscopically by PFU quantification. Viral titers of treated and untreated ZIKV-MR766 aliquots were reported as PFU/mL and compared (Students t-test).

3.2.2.10. *Time-of-addition assays*

To investigate the mechanism of action of ellagic acid, a set of time-of-addition assays was performed using Vero cells pre-seeded in 24-multiwell plates at 1 X 10^5/well density. Serial dilutions of ellagic acid were added to Vero cells before the infection for 2 hours at 37 °C (pre-treatment), or during the infection with ZIKV-MR766 and ZIKV-HPF2013 (MOI 0.005), or after the infection (post-infection). After an incubation period of 72 hours in 1.2%-methylcellulose with serial dilutions of ellagic acid, the cells were fixed and stained, and viral plaques were counted. Results were reported as percentages of viral plaques in comparison to controls for each concentration of ellagic acid, and the EC_{50} values were determined.

3.2.2.11. *Statistical analysis*

Data were analyzed using Student's t-test or F-test by GraphPad Prism version 5.00 software, as appropriate. The Student's t-test was used to compare viral titers in virus inactivation assays. Significance was reported for p-value <0.05.

3.2.3. Anticancer evaluation assays on acute lymphoblastic leukemia (ALL) and multiple myeloma (MM) cells

3.2.3.1. Preliminary cytotoxicity assays on ALL cells

Ethanolic pomegranate summer leaves extract (PGSumTot), autumn leaves extract (PGAutTot), SPE fractions methanol/water 85:15 (PGAut85, PGSum85) and methanol/water 95:05 (PGAut95, PGSum95) were dissolved in DMSO at concentration of 20 mg/mL (Stock solution). Several pure compounds identified in the raw material such as Ellagic acid, Betulinic acid, Oleanolic acid, Ursolic acid, Quercetin, Luteolin 4'-*O*-glucoside, Quercetin 3-*O*-glucuronide, Quercetin 3-*O*-glucoside, Apigenin 7-*O*-glucoside, were dissolved in DMSO at concentration of 20 mM (Stock solution). Extracts, SPE fractions and pure compounds solutions were subsequently diluted in Roswell Park Memorial Institute (RPMI) 1640 medium at 20 µg/mL and 20 µM respectively and added (100 µL) in ratio 1:1 to two acute lymphoblastic leukemia (ALL) cell lines: a drug-sensitive CCRF-CEM and its derived multidrug-resistant, P-glycoprotein over-expressing CEM/ADR5000 obtained by continuous treatment with doxorubicin up to a concentration of 5000 ng/mL as described by Kimming et al. [44], both at 1x10^4 cells/well cultured in RPMI 1640 medium, supplemented with 10% fetal bovine

serum (FBS) and 1% penicillin (100U/ml)-streptomycin (100 µg/ml) antibiotic, in a 96-well plate and incubated in humidified 5% CO_2 atmosphere at 37°C for 72h. After the incubation time, the resazurin reduction assay was performed to assess the cytotoxicity of the samples/compounds toward drug-sensitive and resistant cell lines. The non-fluorescent dye resazurin is reduced metabolically by living cells to the strongly fluorescent dye resorufin. 20 µL of 0.01% w/v resazurin (Sigma-Aldrich, Schnelldorf, Germany) was added to each well. Cells were incubated for 4 h at 37°C. Fluorescence at excitation wavelength of 544 nm and emission at 590 nm was measured using Infinite M2000 ProTM plate reader (Tecan, Crailsheim, Germany). The percentage of viable cells was calculated as follows: Cell Viability (% of control) = (absorption (treated cells) – absorption (medium alone)) / (absorption (untreated cells) – absorption (medium alone)) * 100.

3.2.3.2. Toxicity of ursolic acid on MM cells

Ursolic acid has been tested as a dose response assay (between 10^{-7} and 10^{2} µM) on nine multiple myeloma cell lines: JJN, RPMI8226, AM01, OPM2, KMSII, NCIH929, L363, MolP8, KMS12BM. Doxorubicin was included as a positive control for each experiment.

3.2.3.3. Toxicity of ursolic acid in normal cells

The human peripheral mononuclear cells (PMNC) were isolated from fresh blood samples of a healthy donor with Histopaque® (Sigma-Aldrich, St. Louis, MO, USA). In brief, 3 mL of blood was layered with 3 mL of Histopaque® and centrifuged (400 × g) for 30 min at 4°C. The buffy coat interface, containing lymphocytes and other mononuclear cells, was transferred into a new tube and washed several times. Isolated PMNCs were kept in Panserin 413 medium (PAN-Biotech, Aidenbach, Germany) supplemented with 2% phytohemagglutinin M (PHA-M, Life Technologies, Darmstadt, Germany). Afterwards, the cells were incubated 72h in a 96 wells plate as a dose/response assay (between 10^{-4} and 10^{2} µM) and the resazurin assay was carried out as described above.

3.2.3.4. Cell cycle analysis

To determine whether ursolic acid has an effect on the cell cycle phase distribution, RPMI8226 cells were treated with various IC_{50} concentrations (3.6-14,4 µM) of ursolic acid for 24 h. Afterwards, cells were washed with cold PBS and fixed overnight in ice-cold 70% ethanol (Sigma-Aldrich, Germany). Fixed cells were washed with PBS and incubated with 50 µg/mL propidium iodide (PI, Carl-Roth) in PBS for 5 min in the dark. 1×10^{4} cells were counted for each sample with AccuriTM C6 cytometer (BD Biosciences, Heidelberg, Germany). PI was measured at 488 nm excitation (100 mW) and detected using a 610/20 nm band pass filter. Cell cycle distribution of untreated cells after 24 h were used as control. Cytographs were analyzed using BD Accuri C6 software (BD Biosciences). All experiments were performed three times.

3.2.3.5. Assessment of the mode of action of ursolic acid by Annexin V-PI staining

Annexin V is an intracellular protein that calcium-dependently binds phosphatidylserine (PS), which translocates from the internal leaflet of the plasma membrane to the external leaflet during early apoptosis. Propidium iodide (PI) is excluded by living or early apoptotic cells with intact membranes

and stains late apoptotic and necrotic cells with red fluorescence because of the DNA intercalation. Hence, cells with annexin V (−) and PI (−) are considered to be alive, while cells with annexin V (+) and PI (−) are in early apoptosis. Cells in late apoptosis or necrosis are both annexin V and PI positive [40]. RPMI8226 cells were treated with IC_{50}, $2xIC_{50}$, $3xIC_{50}$, $4xIC_{50}$ of ursolic acid for 48 h. DMSO was tested as vehicle control. After incubation, cells were collected by centrifugation. After washing with PBS, cells were incubated with annexin V and PI binding buffer (Thermo Fisher, Darmstadt, Germany) according to the manufacturer's protocol. Then, 2×10^4 cells were counted and measured with AccuriTM C6 cytometer (BD Biosciences, Heidelberg, Germany). The annexin V-APC signal was measured at 640 nm excitation and detected using a 675/25 nm band passfilter. The PI signal was analyzed at 488 nm excitation and detected using a 585/40 nm band passfilter. All parameters were plotted on a logarithmic scale. Cytographs were analysed using BD Accuri C6 software (BD Biosciences). All experiments were performed in triplicate.

3.2.3.6. Measurement of reactive oxygen species (ROS)

2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFH-DA) (Sigma-Aldrich, Germany) is a probe used for the highly sensitive and quantifiable detection of ROS. The nonfluorescent H_2DCFH-DA molecule diffuses into the cells and is cleaved by cytoplasmic esterase into 2',7'dichlorodihydrofluorescein (H_2DCF) that cannot diffuse back out of the cells. When hydrogen peroxide is present, H_2DCF is oxidized to the fluorescent molecule dichlorofluorescein (DCF) by peroxidases. DCF radiates a fluorescent signal measured and quantified by flow cytometry, and giving an indication of intracellular ROS concentration [34]. RPMI8226 cells were resuspended in 1 ml of PBS and incubated with 10 μM H_2DCFH-DA for 30 min in the dark. For positive control 100 μM H_2O_2 were added and incubated for 15 min in the dark. Cells were then measured with AccuriTM C6 cytometer (BD Biosciences, Heidelberg, Germany). For each sample, 1×10^4 cells were counted. DCF was measured at 488 nm excitation (25 mW) and detected using a 530/30 nm band pass filter. All parameters were plotted on a logarithmic scale. Cytographs were analysed using BD Accuri C6 software (BD Biosciences). All experiments were performed in triplicate.

3.3. Results and discussion

3.3.1. Bioguided leaf extract fractionation and HIV-1 RT/IN inhibition

It is known from current literature the antiviral properties against HIV-1 replication by ellagic acid [14,20], pentacyclic triterpenoid acids [45,46] and flavonoid aglycons such as luteolin [47] and apigenin [48]. A bioguided fractionation of the active ethanolic pomegranate leaf extract, provided by the Department of Life and Environmental Sciences of the University of Cagliari, was performed in order to obtain enriched flavonoid fractions and to evaluate their antiviral potential against HIV-1 RT and IN enzymes.

3.3.1.1. Column chromatography

Gravimetric normal-phase silica gel column chromatography was used to fractionate the leaf crude extract. The resulting three main fractions were considered. Fraction A (44 mg) was obtained joining together the fractions eluted with Pe/EtOAc 5:5 – EtOAc 100% and EtOAc/MeOH 98:2. Fraction B

(37 mg) was eluted with EtOAc 100% and fraction C (28 mg) with EtOAc / MeOH 99:1. The latter was subsequently dissolved in hot acetone and placed in the freezer at -20°C overnight, then the surnantant was recovered and evaporated. Figure 1 reports the LC chromatographic profiles of the fractions while Table 1 and Figure 2 show the list of compounds identified in the fractions and their quantitation by HPLC-PDA-ESI-MS/MS, respectively. Fraction B presented the higher content of ellagic acid while fractions A and C were enriched in flavonoid components. The enriched fractions were then tested for *in vitro* assays to evaluate the HIV-1 RT and IN inhibition.

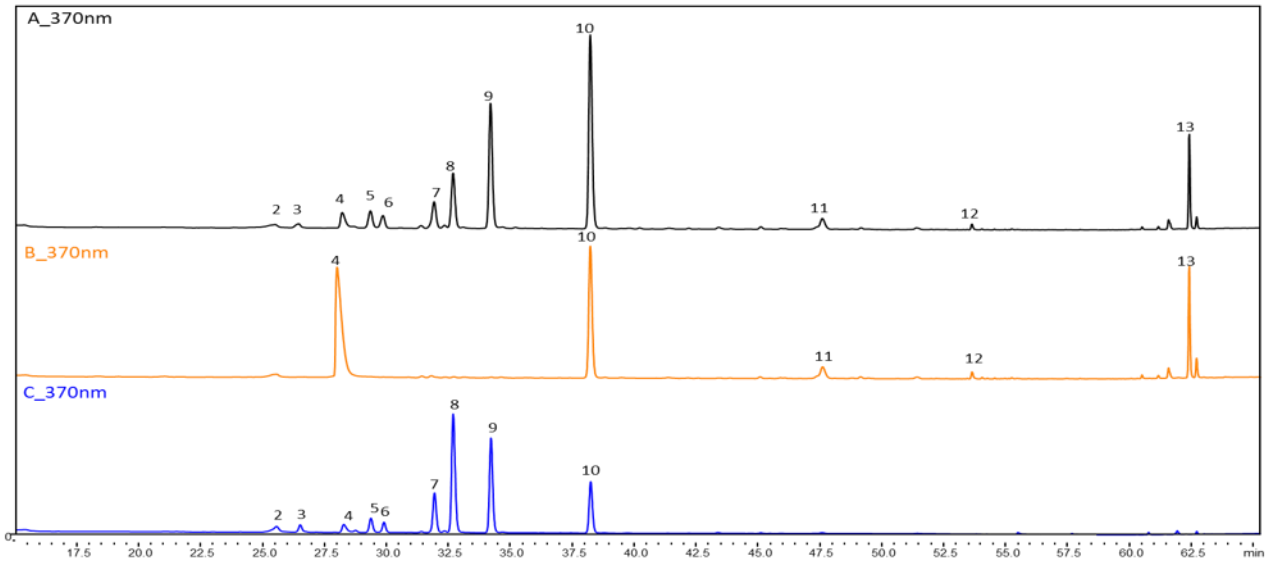

Fig. 1. LC representative chromatograms of the fractions obtained by the crude leaf extract fractionation. Compounds are numbered according to Table 1.

Table 1. List of identified and putatively identified compounds in the fractions.

N°	Compound name
1	Ellagic acid glucoside
2	Galloyl- HHDP-DHHDP-hexoside (Granatin B)
3	Luteolin 7-*O*-glucoside
4	Ellagic acid
5	Quercetin 3-*O*-glucoside
6	Apigenin 7-*O*-glucoside
7	Apigenin glycoside
8	Luteolin 4-*O*-glucoside
9	Luteolin glycoside
10	Luteolin glycoside
11	Luteolin
12	Apigenin
13	Pheophorbide A

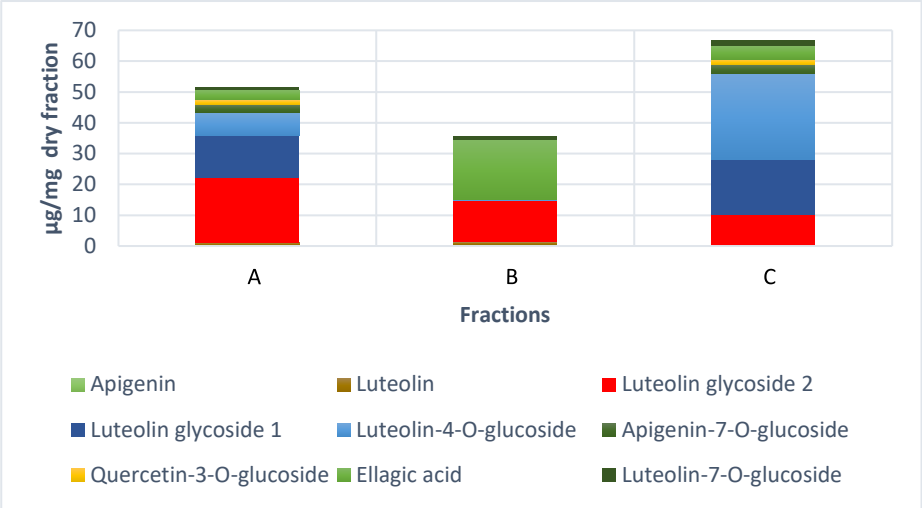

Fig. 2. Quantitation of the main compounds identified in the fractions expressed in µg/mg of dry fraction

3.3.1.2. HIV-1 in vitro antiviral activity

All fractions showed antiviral activity (Table 2) but the total crude extract and ellagic acid strongly inhibited the IN enzyme, which represents the main target of the latest anti-HIV-1 class of drugs [12] with IC_{50} values comparable to the control. The simultaneous inhibition of both RT and IN activities possibly by new mechanisms of action would provide a significant advantage against drug-resistant variants.

Table 2. [a]Extract/Compound concentration required to inhibit the HIV-1 RNase H activity by 50%; [b]Extract/Compound concentration required to inhibit the HIV-1 IN catalytic activities, in the presence of LEDGF, by 50%.

Samples and controls	**HIV-1 RT RNase H IC_{50} (µg/ml) [a]**	**HIV-1 IN LEDGF dependent IC_{50} (µg/ml) [b]**
Crude leaf extract	0.61 ± 0.02	0.12 ± 0.07
A	0.51 ± 0.04	0.88 ± 0.11
B	0.77 ± 0.02	0.67 ± 0.04
C	0.45 ± 0.003	0.66 ± 0.08
Ellagic acid	1.40 ± 0.11	0.08 ± 0.0005
Efavirenz	0.015 ± 0.005	-
Raltegravir	-	0.058 ± 0.01

3.3.2. Bioguided leaf extract fractionation and Zika Virus inhibition

3.3.2.1. Citotoxicity and antiviral evaluation of the total extracts

Since currently no vaccine or treatment is available against Zika virus (ZIKV) infection [19] and within a project aiming to investigate the potential antiviral activity of non-edible parts of plants used in traditional medicine, pomegranate leaf extract, fractions and main components were evaluated for

the potential anti-ZIKV activity. The ethanolic leaf extract from the sample collected in the botanical garden of Cagliari in autumn and from a homogeneous sample of leaves harvested in summer season were tested. After chlorophylls removal by SPE-C18 cartridge, the total extracts were initially tested for their citotoxicity and anti-ZIKV activity by MTS and viral plaque reduction assay, respectively.

Investigations were made on Vero cells by increasing extract concentrations added to the cell culture before the infection to generate dose-response curves (Figure 3). The CC_{50} values for the autumn (PGAutTot) and summer (PGSumTot) extracts were 123.6 µg/ml and 132.2 µg/ml whereas EC_{50} values were 11.40 µg/ml and 7.76 µg/ml with a Selectivity Index (SI) of 10.84 and 17.07 respectively, indicating that the antiviral effect was not a consequence of cytotoxicity (Table 3).

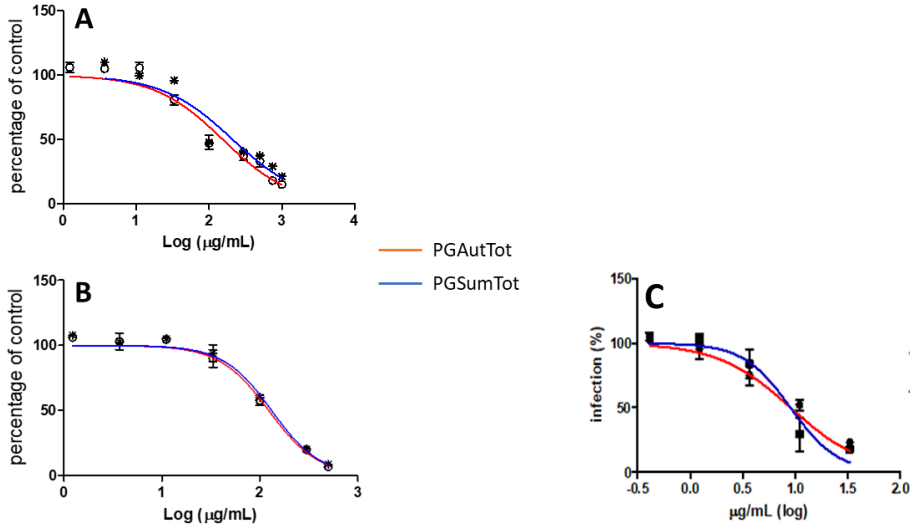

Fig. 3. Cytotoxicity assay (MTS) of autumn harvesting leaves (PGAutTot) and summer harvesting leaves (PGSumTot) on VERO cells at 24h and 72h incubation time (**A**) and (**B**), and antiviral assay on VERO cells infected by ZIKV (**C**).

Table 3. [a]CC_{50}: half maximal cytotoxic concentration at 24h and 72h; [b]EC_{50}: half maximal effective concentration; [c]SI: Selectivity Index of the total extract: summer leaves (PGSumTot) and autumn leaves (PGAutTot); CI[d]: confidence interval. The Selectivity Index (SI) was calculated as the ratio CC_{50} / EC_{50}.

Extract	CC_{50}[a] 24h (µg/mL) (95% CI[d])	CC_{50}[a] 72h (µg/mL) (95% CI[d])	EC_{50}[b] (µg/mL) (95% CI[d])	Selectivity Index (SI)[c]
PGAutTot	154.9 (112.3-213.7)	123.6 (104.0-146.7)	11.40 (7.48-16.57)	10.84
PGSumTot	211.9 (145.5-308.8)	132.2 (113.2-154.4)	7.76 (5.62-10.70)	17.03

3.3.2.2. Extract fractionation

To attribute the antiviral activity to a specific fraction and/or to single components of pomegranate leaf extract, a bio-guided fractionation procedure was performed. 30 mg of each ethanolic extract, were submitted to SPE fractionation resulting in two fractions: a phenolic fraction (22 mg) eluted

with methanol/water 85:15 (PGAut85, PGSum85) and a triterpenoid fraction (1.5 mg) eluted with methanol/water 95:05 (PGAut95, PGSum95). Figure 4 reports the main compounds detected and quantified by HPLC-PDA-MS/MS system for phenols and GC-MS system for triterpenes. The phenolic fraction was characterized by ellagic acid, rutin, apigenin, quercetin and luteolin glycosides. In the fraction obtained from the autumn sample (PGAut85), ellagic acid was the most abundant compound while in the the summer sample (PGSum85), luteolin 4'-*O*-glucoside was the main compound. The triterpenoid fraction was characterized by oleanolic, betulinic and ursolic acids. In the fraction obtained from the summer pool (PGSum95), triterpenoids were more abundant that the same fraction obtained from the autumn sample (PGAut95).

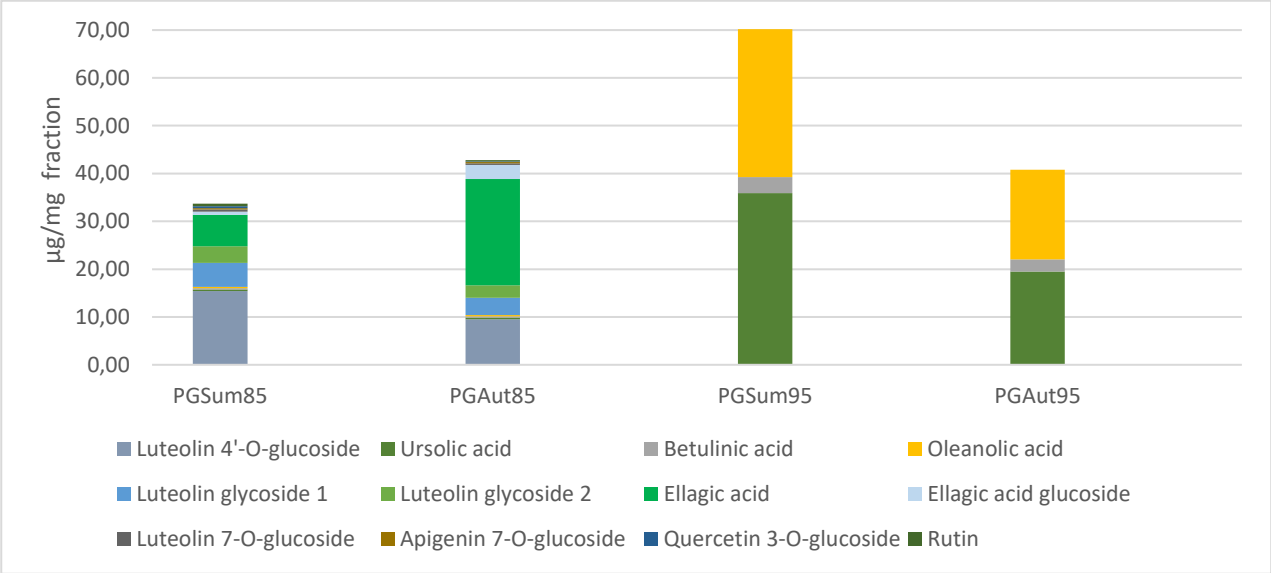

Fig. 4. Quantitation of the main compounds identified in the fractions; phenolic fraction eluted with 85:15 Methanol/water (PGSum85 and PGAut85) and terpenoid fraction eluted with 95:05 Methanol/water (PGSum95 and PGAut95)

3.3.2.3. ZIKV antiviral activity of fractions

Viral plaque reduction assay on the different fractions showed that only the phenolic fractions were active with an EC_{50} of 10.37 and 16.20 µg/mL respectively for PGAut85 and PGSum85 (Table 4 and Figure 5). In addition, all the compounds identified in the phenolic fraction were tested as commercial standards and only ellagic acid as commercial standard showed antiviral activity therefore a further step of fractionation was included to isolate ellagic acid from the phenolic fractions.

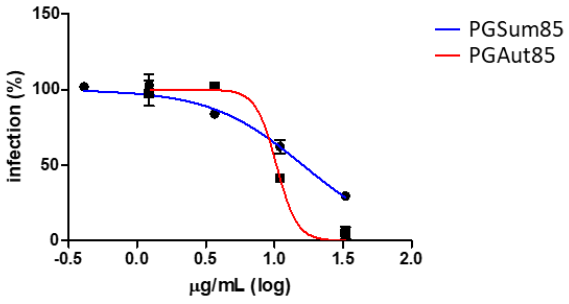

Fig. 5. Antiviral assays of phenolic fractions derived by: Cagliari botanical garden autumn harvesting leaves (PGAut85) and pool of summer harvesting leaves (PGSum85) on VERO cells infected by ZIKV.

Table 4. [a]CC_{50}: half maximal cytotoxic concentration; [b]EC_{50}: half maximal effective concentration; [c]SI: Selectivity Index of the phenolic fractions; CI[d]: confidence interval.

Extract	CC_{50}[a] 72h (µg/mL) (95% CI[d])	EC_{50}[b] (µg/mL) (95% CI[d])	Selectivity Index (SI)[c]
PGAut85	73.4 (51.6-104)	10.4 (7.08-15.2)	7.05
PGSum85	76.1 (48.4-120)	16.20 (12.4-21.2)	4.69

3.3.2.4. LC-Prep purification of ellagic acid

PGAut85 fraction resulting in a lower EC_{50} value and in a higher ellagic acid content was selected for the isolation of the compound by LC-Prep. Figure 6A reports the ^{1}H NMR of the purified ellagic acid compared to the ^{1}H NMR of commercial standard used as reference (Figure 6B). The molecule isolated showed a HPLC Purity grade: >97% at 254 nm.

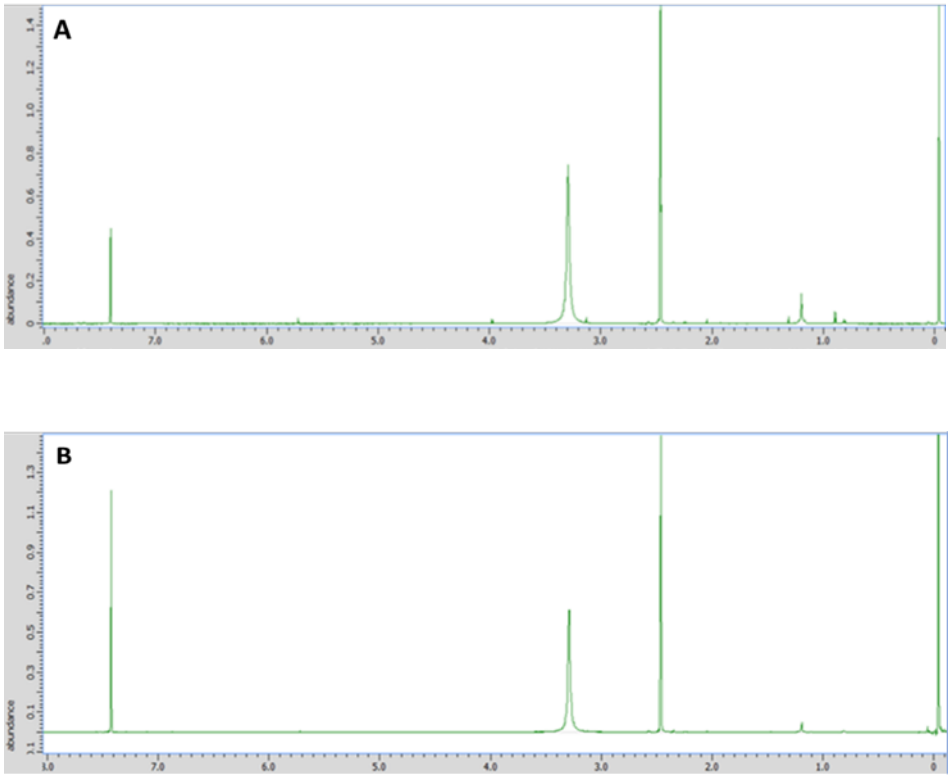

Fig. 6. ^{1}H 600 MHz NMR of ellagic acid isolated with LC-Prep (**A**) and its commercial standard reference (**B**).

3.3.2.5. ZIKV antiviral activity of ellagic acid

Two ZIKV strains were used to investigate the antiviral potential of ellagic acid: the 1947 Uganda MR766 and the 2013 French Polynesia HPF2013, representing the African and the Asian lineage respectively. Figure 7 and Table 5 report the results of the dose-response curves: the antiviral assay against ZIKV MR766 strain with EC_{50} value of 8.38 µg/ml (A) and against ZIKV HPF2013 with EC_{50} value of 6.33 µg/ml (B) suggest that the antiviral effect was not a consequence of cytotoxicity. The results are reported for three experiments performed in duplicate.

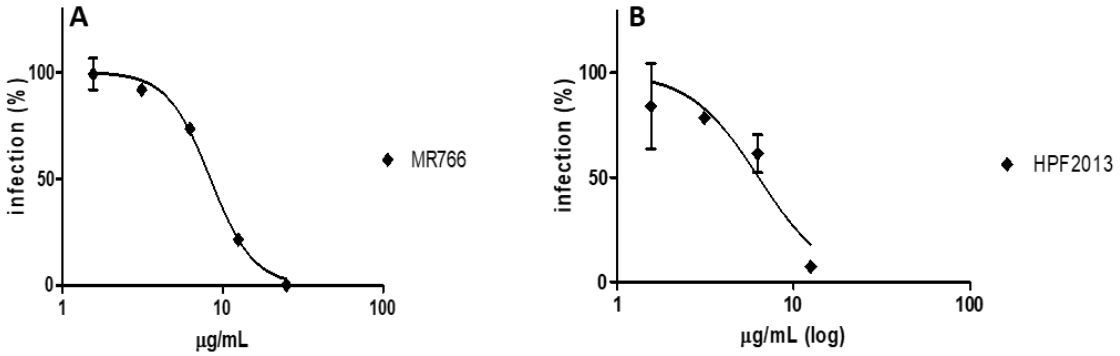

Fig. 7. Antiviral assay of ellagic acid against ZIKV MR766 an HPF2013 strains (**A**) and (**B**), respectively.

Table 5. [a]CC_{50}: half maximal cytotoxic concentration; [b]EC_{50}: half maximal effective concentration; [c]SI: Selectivity Index of ellagic acid on the two ZIKV strains tested; CI[d]: confidence interval.

ZIKV strain	CC_{50}[a] 72h (µg/mL)	EC_{50}[b] (µg/mL) (95% CI[d])	Selectivity Index (SI)[c]
MR766	150	8.38 (7.47-9.41)	17.89
HPF 2013	150	6.33 (4.57-8.78)	23.69

3.3.2.6. Virus inactivation assay

To explore whether the ellagic acid directly inactivates ZIKV particles, a virus inactivation assay was performed. Dilutions from both the ZIKV-MR766 sample treated with ellagic acid and untreated control were titrated to non-inhibitory concentrations on Vero cells. After 72-hour incubation at 37°C, the cells were fixed and stained, and the viral plaques were counted. Viral infectivity was determined microscopically by PFU quantification.

The ZIKV-MR766 experiment is reported here as an example: this treatment did not produce a significant loss of ZIKV infectivity proving that ellagic acid does not perform a direct effect on viral particles. This result indicates that the ellagic acid most probably targets cell-surface or intracellular events involved in fundamental steps of the ZIKV replicative cycle. Therefore, to investigate which

step(s) of the virus replicative cycle was affected by the ellagic acid, a time-of-addition assay was run.

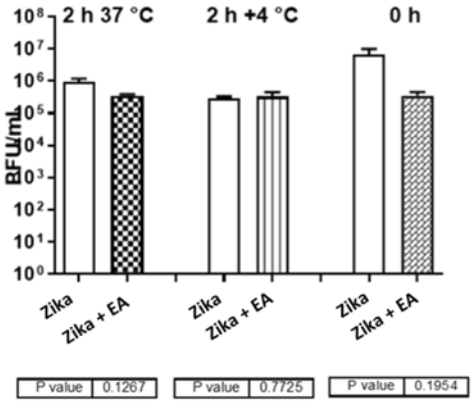

Fig. 8. Virus inactivation assay: a suitable amount of ZIKV particles (10^5 pfu) as described by [43] were incubated with ellagic acid concentrations corresponding to EC_{90} values (µg/mL) as obtained in the viral plaque reduction assay for 0 or 2 h at 37˚C or 4°C. Viral titers of treated and untreated ZIKV-MR766 aliquots were reported as PFU/mL (Students t-test).

3.3.2.7. Time of addiction assay

To investigate the mechanism of action of ellagic acid, a set of time-of-addition assays was performed using Vero cells. Serial dilutions of ellagic acid were added to Vero cells before the infection for 2 hours at 37 °C (pre-treatment), or during the infection with ZIKV-MR766 and ZIKV-HPF2013, or after the infection (post-infection). Results showed that ellagic acid was active only when added to the cells before virus inoculum (pre-treatment assay) (Fig. 9A); a significant suppression of ZIKV replication was observed with an EC_{50} value of 19.5 µg/ml (95% CI: 16.1 to 23.6). No inhibition was observed when the ellagic acid was added during (9B) and after the infection (9C). Taken together, the results from the virus inactivation and time-of-addition assays show that ellagic acid exerts its antiviral activity by inhibiting first steps of the virus replicative cycle (e.g. adsorption, penetration, and primary transcription) while it does not affect the later steps (e.g. uncoating, genome replication, virus assembly or exit, cell-to-cell spread). This could suggest a possible use of ellagic acid as a drug in prophylaxis.

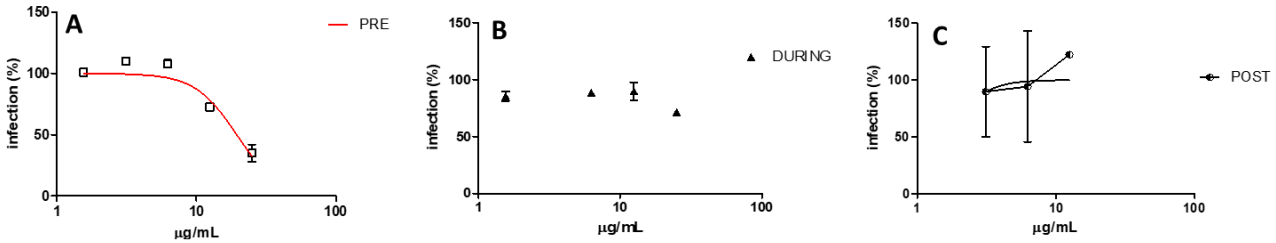

Fig. 9. Time-of-addition assays with ellagic acid: A) Vero cells were treated with prior to virus infection (pre-treatment); B) during the infection period (during infection) and C) after infection (post-treatment).

3.3.3. Anticancer evaluation assays on in vitro acute lymphoblastic leukaemia and multiple myeloma cells

3.3.3.1 Preliminary cytotoxicity assays on acute lymphoblastic leukaemia cells

A preliminary screening to assess the cytotoxic potential of pomegranate ethanolic leaf extracts, fractions and pure compounds (10 µg/µL and 10 µM solution respectively) was performed against human leukaemia drug-sensitive CCRF-CEM cells and P-glycoprotein multidrug-resistant CEM/ADR5000 *in vitro* developed subline. After the treatment exposure for 72 hours, the viability of the cells was quantified by using resazurin colorimetric assays as reported by Mahmoud et al. [34]. Figure 10A showed that among all the tested extracts, fractions and pure compounds, ursolic acid exhibited the highest cytotoxic effect on CCRF-CEM leukaemia cells comparable with those of positive controls (Doxorubicin, Daunorubicin and Epirubicin). Betulinic and ursolic acids showed the highest cytotoxic potential compared to other molecules on multidrug-resistant P-glycoprotein (ABCB1/MDR1)-overexpressing CEM/ADR5000 cells (Fig. 10B). These results are in accordance with their anticancer potential reported in literature [49,50].

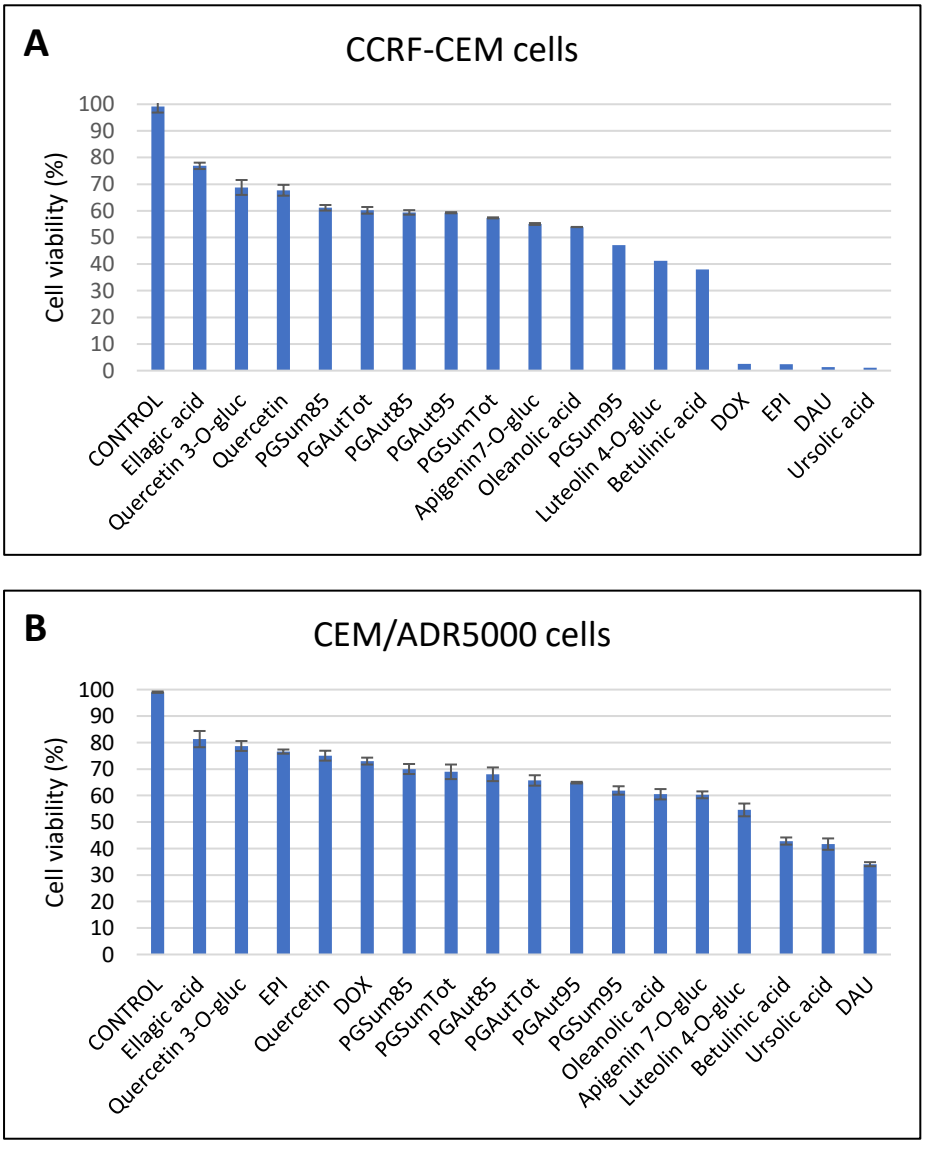

Fig. 10. Cytotoxicity assays performed using resazurin reduction assay in acute lymphoblastic leukaemia (ALL) cell lines:

(**A**) drug-sensitive CCRF-CEM and (**B**) multidrug-resistent CEM/ADR5000 of PG total leaf extract harvested in autumn (**PGAutTot**), in summer (**PGSumTot**), their respectively SPE-C18 fractions MeOH/water 85:15 (**PGAut85, PGSum85**), MeOH/water 95:5 (**PGAut95, PGSum95**) at the concentration of 10 µg/ µl, and pure compounds: ellagic acid, betulinic acid, oleanolic acid, ursolic acid, luteolin 4'-*O*-glucoside, quercetin 3-*O*-glucoside, apigenin 7-*O*-glucoside and controls (**DMSO**), Doxorubicin (**DOX**), Epirubicin (**EPI**) and Daunorubicin (**DAU**) (10µM) at the concentration of 10 µM.

3.3.3.2. Toxicity of ursolic acid on multiple myeloma cells

On the basis of literature data on the evaluation of the activity of ursolic acid on human multiple myeloma cell lines U266, RPMI 8226, and MM1.S [38,51], its anticancer potential was extended to more human multiple myeloma cell lines (JJN, RPMI8226, AM01, OPM2, KMSII, NCIH929, L363, MolP8, KMS12BM). Dose/Response assays between concentrations of 10^{-7} and 10^{2} µM were performed and after 72h incubation, the resazurin assay was carried out. A conventional chemotherapeutic agent such as doxorubicin, was used as control. Figure 11 and Table 6, report a good cytotoxic response of ursolic acid with IC_{50} between 2 and 10 µM/mL in all the tested cell lines. In addition, ursolic acid showed no citotoxicity on human peripheral mononuclear cells (PMNC) isolated from fresh blood sample of a healthy donor (Figure 12). This result is supported by a study conducted by [52], pointing out that ursolic acid selectively kills human leukaemia cell lines but not normal hematopoietic cells. On the other hand, Doxorubicin exhibited very high cytotoxicity on all the cell lines tested with IC_{50} between 0.0012 and 0.053 µM/mL; up to now it is used as a typical anticancer agent in common chemotherapy but beside its several side effects, especially cardiomyopathy [53], and toxicity on normal blood cells [54], it induces the expression of MDR genes which involves a complexity of the cancer treatment, difficult to overcome [33]. Therefore it is necessary to find new chemotherapeutic agents. For these reasons, one of the most responding cell lines to ursolic acid cytotoxicity (RPMI8226) was chosen for further investigation.

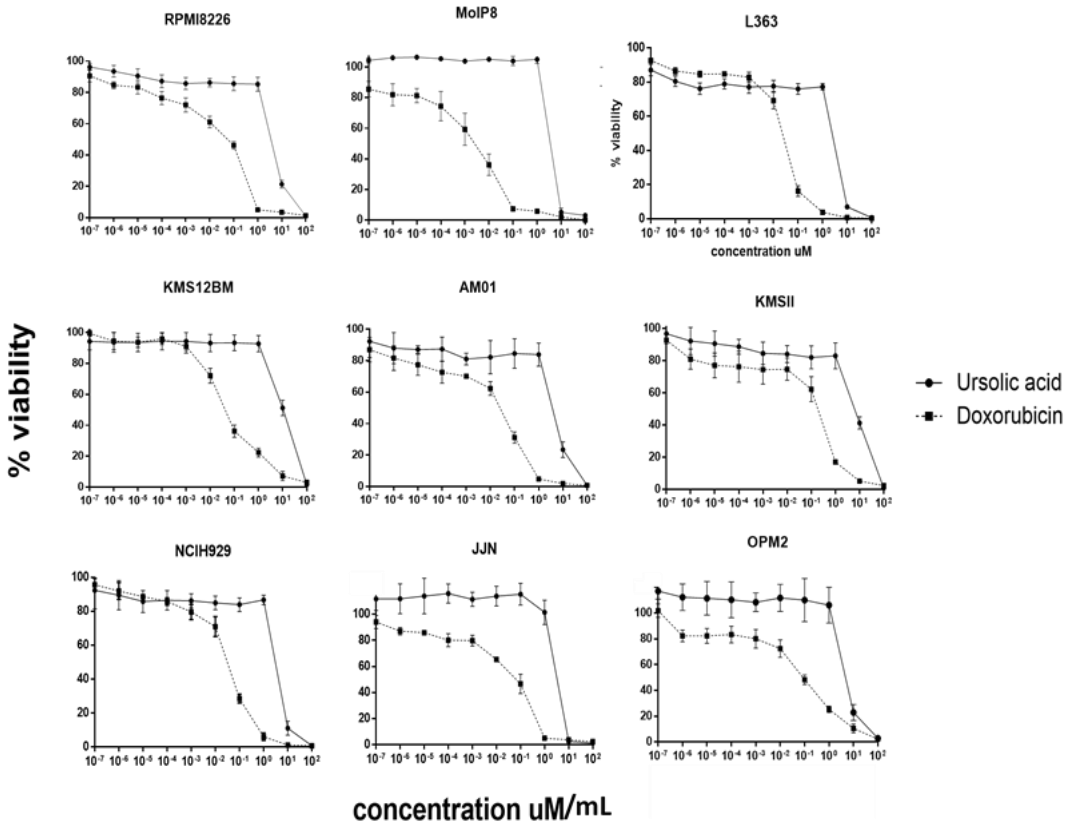

Fig. 11. Cytotoxicity of ursolic acid toward multiple myeloma cell lines (RPMI8226, MolP8, L363, KMS12BM, AM01, KMSII, NCIH929, JJN, OPM2) as determined by resazurin assays.

Table 6. Concentrations (μM) of ursolic acid and doxorubicin required to inhibit the cells viability on multiple myeloma cells by 50%.

Cell line tested	Ursolic acid IC_{50}(μM/mL)	Doxorubicin IC_{50} (μM/mL)
RPMI8226	3.618 ± 0.329	0.0128 ± 0.004
MOLP8	6.895 ± 0.263	0.0012 ± 0.0002
L363	2.124 ± 0.138	0.0175 ± 0.005
KMS12BM	10.03 ± 1.456	0.0519 ± 0.013
AM01	3.608 ± 0.626	0.0058 ± 0.003
OPM2	8.065 ± 1.639	0.0534 ± 0.043
NCIH929	2.954 ± 0.073	0.0224 ± 0.008
JJN	5.874 ± 1.759	0.0249 ± 0.009
KMSII	5.320 ± 1.626	0.0430 ± 0.013

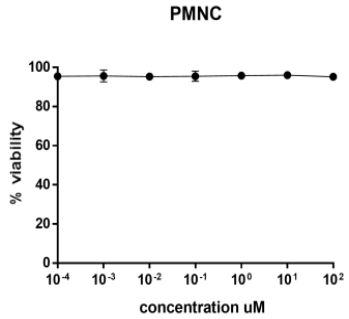

Fig. 12. Cytotoxicity of ursolic acid toward human peripheral mononuclear cells, as determined by resazurin assays.

3.3.3.3. Cell cycle analyses by flow cytometry and PI staining

The analysis of cell cycle distribution is important for studying cell growth differentiation, senescence and apoptosis and the earliest and simplest approach to perform it, is represented by measuring of cellular DNA content with a fluorescent DNA dye (e.g., propidium iodide), that differentiates resting/quiescent cell populations (G0 cell) and quantifies cell cycle distribution at a single phase (i.e., G0/G1 (2n), S (2n-4n), and G2/M (4n)) [55]. The RPMI8226 cells were treated with ursolic acid, revealing that the cell cycle phases were modified in a concentration-dependent manner (Figure 13). Increase of cells in sub-G0/G1 and G2/M phases was observed. The percentage of RPMI8226 cells in sub-G0/G1 phase in nontreated cells was 8.2%; meanwhile, it varied upon treatment from 13% (IC_{50}) to 14% (2 × IC_{50}), 16% (3 × IC_{50}) to 26% (4× IC_{50}). These results suggest that ursolic acid caused cycle arrest in G2/M phase in RPMI8226 cells.

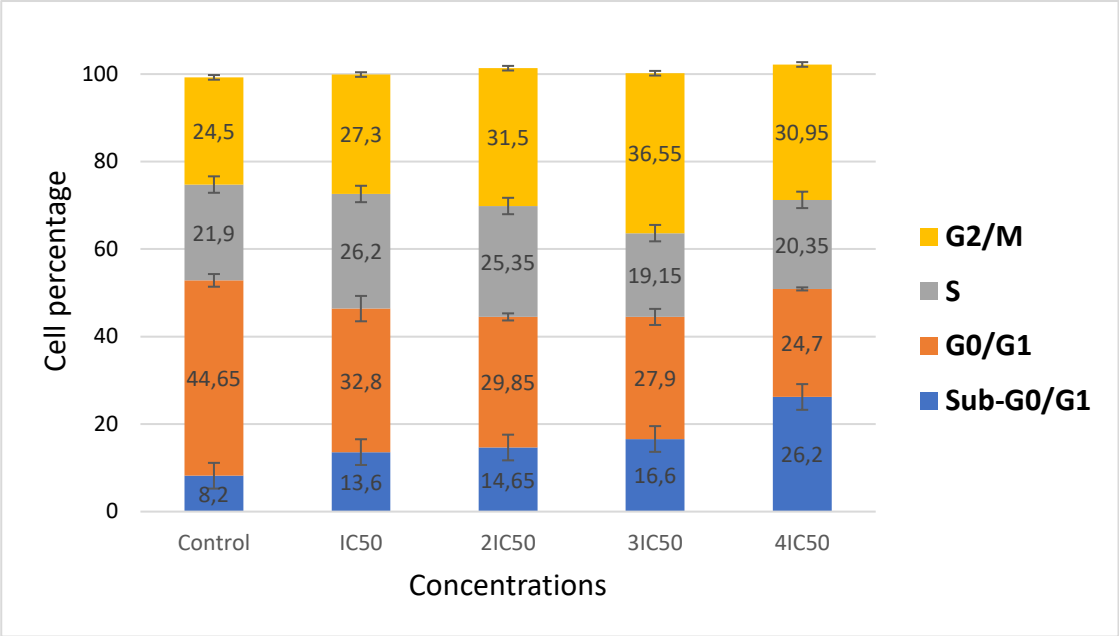

Fig. 13. Distribution of RPMI8226 cell cycle upon 24h treatment with ursolic acid. IC_{50} values were 3.6, 7.2, 10.9, 14.4 µM/mL.

3.3.3.4. Apoptosis evaluation by annexin V-PI staining

Flow cytometry-based methods providing the combination of Annexin V and Propidium iodide (PI) are commonly used to analyse differences in plasma membrane permeability and integrity, establishing if cells are viable, apoptotic or necrotic. This approach is widely used for studying apoptotic cells. PI is a good, economical and stable indicator of cell viability due to the fact that it can pass through the damaged plasma membrane of late apoptotic and necrotic cells, intercalating nucleic acids and showing red fluorescence, while it cannot stain viable or early apoptotic cells because of an intact plasma membrane. Annexin V is a protein that binds phospholipids such as phosphatidylserine (PS) which translocates from the inner side of the plasma membrane to the outer layer of cells in the early stages of apoptosis, allowing the binding with the protein and cell staining with fluorescein isothiocyanate (FITCH-labelled Annexin V) and showing green fluorescence. Even if in necrosis, where the cell membrane losses its integrity with the leakage of cellular constituents, Annexin can bind phospholipids. The combination of a dye exclusion test using PI provides a perfect assay in order to discriminate among apoptosis and necrosis [56,57]. However, a limit of this protocol is represented by a positive double Annexin V/PI staining for both cells at the end-stage of apoptosis (late apoptotic) and primary necrotic cells and in order to check the stages of apoptosis, supplementary method as caspase assays are needed [58].

The results of Annexin V/PI staining is reported in Fig.14. The results showed a dose-dependent induction with ursolic acid. When cells were treated with 3 × IC_{50}, for example, ursolic acid induced apoptosis with 62.9% early apoptotic V (+)/PI (-) cells, 10.9% late apoptotic V (+)/PI (+) cells and 0.2% necrotic V (-)/PI (+) cells. These data, coherently with current literature [51], suggested that ursolic acid induced apoptosis instead of necrosis in RPMI8226 cells in a dose-dependent manner.

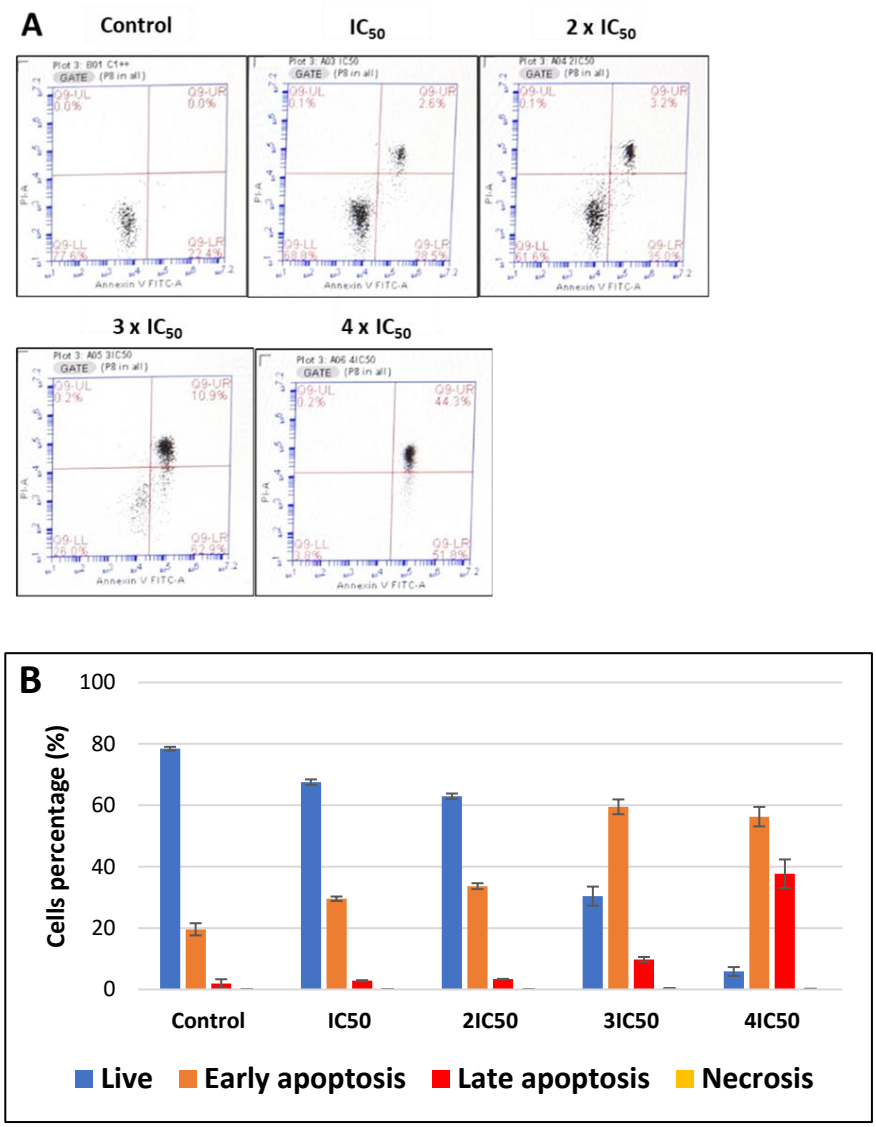

Fig. 14. Evaluation of apoptosis induced by ursolic acid on RPMI8226 multiple myeloma cells after 48h as determined by annexin V/PI assay. Apoptosis was assessed by flow cytometry after annexin V-PI double staining. IC_{50} values were 3.6, 7.2, 10.9, 14.4 µM/mL. Q9-LL: viable cells exhibit annexin V (-)/PI (-); Q9-LR: early apoptotic cells exhibit annexin (+)/PI (-); and Q9-UR and Q9-UL: late apoptotic cells or necrotic cells exhibit annexin V (+)/PI (+) or annexin V (-)/PI (+). Dot plots of Annexin V/PI flow cytometry analysis (**A**). Cells percentage in the different cellular conditions (**B**).

3.3.3.5. *Evaluation of the production of reactive oxygen species (ROS)*

ROS group includes reactive molecules and free radicals deriving from cellular aerobic metabolism. Their high reactivity with biological molecules is influencing the cell functions in response to a different stimuli which can be intracellular or extracellular as drug interaction [59]. To evaluate the possible induction of cytosolic ROS generation mediated by ursolic acid and on the basis of literature data reporting the production of ROS involved in apoptotic cell death [60], the ROS quantification assay using a fluorescent dye as reported by Mahmoud et al. [34] was performed in RPMI8226 cells. Results showed that ursolic acid did not significantly affect the production of ROS in RPMI8226 cells (Figure 15), showing that ursolic acid induced cell apoptosis is independent of ROS pathways as suggested by Chen et al. [61].

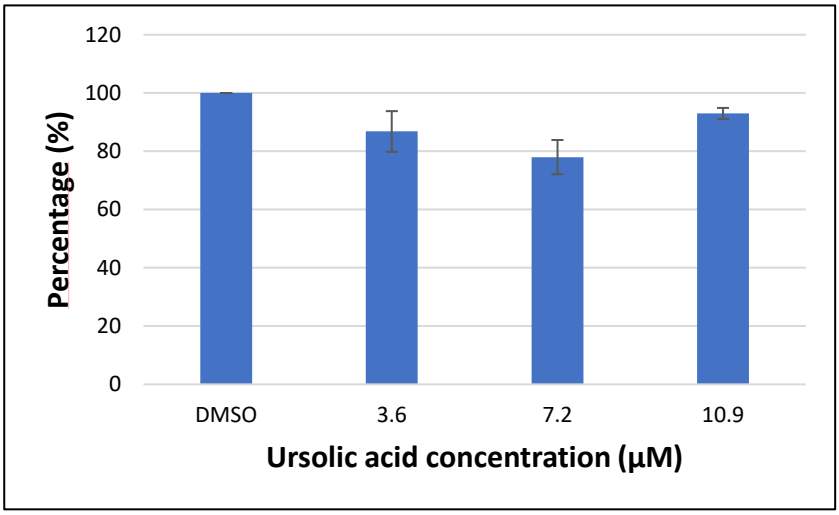

Fig. 15. Effects of ursolic acid (UA) on reactive oxygen species (ROS) compared to the DMSO control in RPMI8226 cells . RPMI8226 cells (1×10^6 cells/well) were incubated with UA (IC_{50}, $2xIC_{50}$ and $3xIC_{50}$) for 24 h for and were measured for ROS. *Significantly different from the control group at $p<0.05$ by one-way ANOVA.

3.4. Conclusions

The leaves of pomegranate have medicinal properties as documented in traditional medicine and represent a rich source of bioactive molecules belonging to the specialised metabolism. The present chapter showed the antiviral and the anticancer potential of the pomegranate ethanolic leaf extract, its fractions and compounds. Nowadays, in order to become more selective against different virus and host cell targets, several anti-HIV chemotherapeutics have been used following various strategies: inhibiting viral entrance into cells by targeting the cell CD4 receptor or cellular CXCR4 or CCR5 co-receptors; inhibiting virus–cell fusion by targeting viral glycoprotein gp41; inhibiting virus adsorption to the host cells by targeting viral envelope glycoprotein gp120; inhibiting HIV retro transcriptase (RT) or HIV integrase (IN) or HIV protease (PR) by targeting their active site; or acting by targeting cellular transcriptional factors [62,63].

Although several anti-HIV drugs have been developed, many troubles such as multidrug-resistant strains and harsh adverse effects have challenged the medical management of HIV infection. HIV needs three essential enzymes for its replication, namely, RT, PR and IN. Most of the anti-HIV drugs commercially available are inhibitors of HIV RT and PR enzymes. HIV IN represents a relatively newer therapeutic target; moreover because the absence of a similar enzyme in the human body, HIV-1 IN is an attractive target for reducing off-target side effects [12]. Since preliminary assays revealed the *in vitro* inhibition of HIV-1 RT and IN by the pomegranate leaf ethanolic crude extract, a bioguided fractionation on gravimetric normal-phase silica gel column chromatography was performed and flavonoid enriched fractions were obtained.

Results showed that all fractions exhibited antiviral activity but the total extract and ellagic acid strongly inhibited both HIV-1 enzymes and in the case of IN results were comparable to the drug control raltegravir which is the first IN inhibitor (INI) for human use [64]. The capability of ellagic acid to inhibit the key functional HIV-1 enzymes is in accordance with literature data [14,15]. In conclusion, in order to overcome the problems associated with viral resistance, up to now the dual inhibitors targeting the HIV-1 RT RNase H and IN which share a common catalytic site residues and geometry, represent the most promising class of HIV-1 inhibitors due to their capability to interact with two targets. As found for pomegranate juice by Neurath et al. [13], the leaf extract deserves further *in vitro* and *in vivo* studies to confirm these findings. It could be foreseen to elude some side

effects associated with antiretroviral drugs including cytotoxicity, investments and time needed for expensive large-scale production. By its part, ellagic acid may have the potential to be developed as a novel agent against HIV-1.

In addition, to enlarge the viral target, ZIKV infection which represents a huge challenge to the global health was considered. Up to now no treatment or vaccine against ZIKV infection is available and the best way to prevent the viral infection is to avoid mosquito bites while analgesics and antipyretics are palliative cures used to treat infected patients, then the research for efficient antivirals is urgently needed [19]. In this context, pomegranate ethanolic leaf extracts obtained from a reference sample collected in autumn in the botanical garden of Cagliari and from a pool of leaves collected in summer season were tested to evaluate the antiviral potential against two ZIKV strains: the 1947 Uganda MR766 and the 2013 French Polynesia HPF2013, representing the African and the Asian lineage respectively. Since results on total extracts showed an antiviral effect without host cell cytotoxicity consequence and good SI index, a bioguided fractionation of the total extracts performed on SPE-C18 cartridge allowed to obtain a phenolic and a triterpenoid fractions. From the phenolic active fraction obtained by the autumn leaves sample which showed an higher content of ellagitannins, ellagic acid was purified using LC-Prep chromatography. In conclusion, pomegranate leaf ethanolic extract, its phenolic fraction and ellagic acid were here shown to be active against ZIKV without host cell citotoxicity. Further studies are needed to elucidate the mechanism of antiviral action in order to identify the cellular and/or viral targets. In addition, results showed that ellagic acid which is the most promising compound in pomegranate leaves against ZIKV, is not active directly on the viral particle and inhibits the first step of the viral replicative cycle which take place before the virus attachment and entry. This represents an interesting feature suggesting additional studies to evaluate its antiviral potential in *in vivo* experiments, especially for its use in prophylaxis. Finally, since specific antivirals against ZIKV are not available, ellagic acid might be an interesting starting point for the development of novel and efficient antiviral pharmaceutical.

Since literature reports promising results attributable to pomegranate and its non-edible part extracts in cancer therapy [3,8]; the last step of this chapter was to evaluate the citotoxicity of the extracts, fractions and pure compounds against cancer cells. Nowadays, the core of the therapy of hematopoietic cancers such as ALL and MM is represented by the use of multi-agent chemotherapy with anthracyclines as doxorubicin, mitotic inhibitors as vincristine, alkylating drugs as melphalan and cyclophosphamide and corticosteroids with allogeneic stem cell transplantation for eligible patients [33,65]. Despite improvements in clinical oncology, the complete cancer remission is hindered by the development of drug resistance; therefore in order to overcome this condition, the development of novel cytotoxic agents has a very high priority [32].

In this topic results from preliminary *in vitro* assays on ALL cell lines: a drug-sensitive CCRF-CEM and its multidrug-resistant P-glycoprotein-overexpressing subline CEM/ADR5000 cells showed that besides all the samples tested which exhibited a citotoxicity between 80 and 40% in both cell lines, only ursolic acid showed the highest cytotoxic effect on CCRF-CEM leukemia cells comparable with conventional chemotherapeutics used as controls. The obtained data on human multiple myeloma cell lines revealed low IC_{50} values for ursolic acid. It induces cell cycle arrest in G2/M phase and apoptosis in RPMI8226 cells confirming its ability to promote the inhibition of proliferation in according to the literature data [38]. In addition the normal lymphocytes control was not affected by its citotoxicity [52]. Micro array analyses on the RNA extracted from RPMI8226 cells before and after treatment with ursolic acid are still in progress, and the data obtained will lead to increase the current knowledge on down or up regulation of gene expression pattern due to the compounds

interaction [38]. These findings suggest a potential therapeutic activity of ursolic acid for the treatment of multidrug resistant tumors and may be further investigated in order to evaluate its effective mechanism of action.

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Chapter 4

Characterization of phenolic contents and antioxidant evaluation of grapevine green pruning residues as a promising by-product

CHAPTER 4: CHARACTERIZATION OF PHENOLIC CONTENTS AND ANTIOXIDANT EVALUATION OF GRAPEVINE GREEN PRUNING RESIDUES AS A PROMISING BY-PRODUCT

Vitis vinifera L. commonly known as grapevine, represents a very interesting source of secondary bioactive metabolites which can provide numerous benefits on human health. Nevertheless viticulture can produce residues and nowadays it is important to find solutions on environmental pollution problem by reducing the amount of waste, promoting the reuse, the recycling and the recovery of the resources even through by-products utilization. This topic has been addressed in the following article (Manuscript ID: molecules-690980) which contributes to add knowledge on the valorisation of grapevine green pruning residues generated by the spring pruning of vineyards. The description of this topic is a merge between the published parts and the supplementary materials.

Grapevine Green Pruning Residues as a Promising and Sustainable Source of Bioactive Phenolic Compounds

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4.1. Introduction

Wine production and, thereby, grape crops are one of today's main and most widespread agro-economic activities, with more than seven million hectares cultivated and 77 million tons produced worldwide in 2016 [1]. Unfortunately, viticulture produces huge amounts of residues, which are a serious economic issue; they are mainly destined to composting or discarded in open areas, potentially causing environmental problems [2]. Solutions involving reuse, recycling, and recovery of resources should, therefore, be found to reduce the amount of waste. The "waste" issue related to environmental sustainability has recently been the object of attention from several regulatory institutions (e.g., Directives 1999/31/EC and 2008/98/EC from the European Commission). The exploitation of by-products generated by grape crops is, therefore, currently of great interest to reduce the environmental impact of wine production, as well as meet the growing demand for green materials and renewable sources of nutrients and bioactive compounds for the feed, functional food, and food supplement industries [3–7].

In the Mediterranean area, and in Italy in particular, wine production and vineyard cultivation are widespread, and result in large amounts of pruning residues waste. In 2011, 3218 million hectares were cultivated as vineyards in Europe [8] of which 736 million hectares were in Italy, for a production of 7487 million tons of grapes [9]. Referring to European data, potential residues derived from grapevine cultivation are estimated to be about 1.4 million tons of dry matter (DM) [10]. Sánchez et al. [11] estimated the amount of vineyard pruning residues in a range of 1 to 7.5 t/ha. The most vigorous pruning is in late summer, but the selective removal of grapevine leaves (together with fruitless young twigs) during spring also generates large amounts of interesting by-products.

This operation is carried out to ventilate and improve the lighting of grape bunches during ripening and consists of removing some or all of the leaves present at the level of the basal area of the shoots, where a lot of bunches are present. The removal process results in a noticeable increase in the content of anthocyanins and other flavonoids [12]. These by-products are known as "green pruning residues" (GPRs) [13]. Many grapevine by-products, such as grape pomace, seeds, and stems, have been characterized in depth, in terms of both chemical composition and biological properties [14–22]. The same is true for vine leaves; these by-products are traditionally used for human and animal consumption, as food [23], animal feed [24], ingredients of dietary supplements [25], and in cosmetics [26]. As for other parts of the grapevine, leaves are mainly characterized by phenolic compounds as specialized metabolites; in particular, phenolic acids, flavonols (mainly in the form of O-glycosides of quercetin and kaempferol) and, to a lesser extent, by stilbenes (resveratrol), flavan-3-ols, and anthocyanins (mainly in red autumn leaves) [7,23,27,28]. The beneficial properties of grapevine leaves are attributed to these phenolic compounds and are principally correlated to the well-known antioxidant activity [7,17,25,29–31].

One of the main limitations to re-using the leaves as by-products is that they are harvested in late summer, after potential treatment(s) with pesticides. Such treatments are a matter of concern for animal feed, because they could induce toxic effects [32]. Conversely, GPRs are harvested during the spring before any treatment has been applied, and therefore are free of pesticide residues, and therefore can be considered as potential health by-products to be exploited. However, to the best of the authors' knowledge, no information concerning the phytochemical phenolic composition or antioxidant properties of these by-products is available. GPRs should not be confused with the "vine pruning residues" that are collected during winter, and therefore are a woody material with a completely different composition (i.e., cellulose, lignin, and other phenolic compounds) [4,33].

Taking into account the above considerations, this study aims to investigate the potential of grapevine green pruning residues (GPRs) as a source of antioxidants, by evaluating their phenolic composition and comparing the results with those of the already exploited late-summer leaves. These investigations involved several cultivars of *V. vinifera* L., both red and white, harvested in Piedmont (Italy) and used to produce some of the most prestigious wines. The phenolic composition of GPRs and leaves was determined through HPLC-PDA-ESI-MS/MS analysis after their extraction with an ultrasound-assisted extraction (UAE) method, previously optimized through an experimental design approach [34]. The antioxidant properties of the extracts were investigated through colorimetric invitro assays (scavenging of the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and the 2,2'-azinobis-3-ethyl-benzthiazoline-6-sulphonate ($ABTS^{+\bullet}$) radicals) and the results were correlated to those obtained by HPLC and total phenol content assay. The antioxidant properties of the matrices were also confirmed by combining the colorimetric *in vitro* assays offline with HPLC-PDA analysis, to determine which compounds contribute most to the antioxidant activity in terms of radical scavenging abilities [35].

4.2. Materials and methods

4.2.1. Plant material and growth conditions

The plant material was harvested during the 2016 and 2017 growing seasons, in an experimental vineyard located in Piedmont (North West Italy). The climate at this site (293 m a.s.l., 45°03'58"N/7°35'37"E) is temperate subcontinental, characterized by two main rainy periods, in

spring and autumn. During the growing season, total precipitation ranges from 139 mm/month (July) to 76 mm/month (May), and the mean temperature and mean relative humidity are 20.3 °C and 68.6%, respectively. Conventional agronomic management was regularly applied in the vineyard, cultivated in soil taxonomically classified as entisol according to the USDA soil classification system [45] which is sandy soil, low in organic matter, with a moderately alkaline pH. Samples of GPRs and leaves of eleven varieties of red grapevine (*Vitis vinifera* Cvs.: Barbera, Cabernet Franc, Cabernet Sauvignon, Canaiolo Nero, Carignano, Grenache, Lambrusco Salamino, Nebbiolo, Pinot Noir, Sangiovese, and Syrah) and five varieties of white grapevine (*Vitis vinifera* Cvs.: Malvasia Bianca, Moscato Bianco, Sauvignon Blanc, Verdicchio, and Vernaccia) were collected in duplicate from standard vertical trellises, with edging shears (see Table 1). Sampling was done in the morning after dew evaporation, during June for GPRs and September for leaves, and was never carried out on rainy days. Fresh GPRs and leaf samples were immediately frozen and freeze-dried using a lyophilizer (5 Pascal, Trezzano sul Naviglio, Italy), and then ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass through a 1 mm screen, and stored.

Table 1. List of the analyzed samples with their acronyms and sampling year

Cultivar	Code	Sampling year	Cultivar	Code	Sampling year
Nebbiolo	N1 – N2	2016	**Cabernet Franc**	CAB	2017
Barbera	B1 – B2	2016	**Canaiolo Nera**	CAN	2017
Sirah	S1 – S2	2016	**Carignano**	CAR	2017
Grenache	G1 – G2	2016	**Lambrusco Salamino**	LAM	2017
Pinot Nero	PN1 – PN2	2016	**Sangiovese**	SAN	2017
Cabernet Sauvignon	CS1 – CS2	2016	**Malvasia Bianca**	MAL	2017
Moscato Bianco	MOS	2017	**Verdicchio**	VER	2017
Sauvignon Blanc	SAU	2017	**Vernaccia**	VSG	2017

4.2.2. Chemicals

HPLC-grade acetonitrile (LC-MS grade), methanol, ethanol, petroleum ether, formic acid (>98%purity), Folin–Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{TM}), potassium persulphate, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, quercetin, kaempferol, resveratrol, and rutin were supplied by Merck (Milan, Italy). De-ionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Quercetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, kaempferol 3-*O*-glucoside, and hyperoside were supplied by Extrasynthese (Genay Cedex, France). Rhamnetin, isorhamnetin, caftaric acid, and quercetin 3-*O*-glucuronide were from Phytolab (Vestenbergsgreuth, Germany).

4.2.3. Extraction method

Extraction conditions (amount of plant material, type of solvent, solvent volume, and extraction time) were carefully optimized through experimental design (see paragraph 4.3.1). A pool sample of GPRs obtained by mixing an equal amount of all cultivars was prepared to run the experimental design experiments. The optimized extraction conditions were as follows: First, 0.100 g of each

sample was extracted with an ultrasonic bath (Soltec, Sonica S3 EP 2400) operating at 40 KHz with 10 mL of methanol/water (70:30, v/v) for 15 min. The ultrasonic bath temperature was set at 30 °C and checked before and after each extraction. The supernatant was centrifuged at 4500 rpm for 10 min and poured into a separatory funnel together with 5 mL of petroleum ether, to reduce chlorophyll interference, by liquid–liquid extraction. The aqueous layer was evaporated in a rotary evaporator under vacuum to a volume of about 1 mL, at a temperature below 50 °C in order to avoid phenol degradation. The extract was then diluted to 2 mL with methanol and filtered through a PTFE 0.22 µm syringe hydrophilic filter for LC analysis. Extraction was repeated thrice for each sample.

4.2.4. HPLC-PDA-MS/MS analysis and quantification

4.2.4.1. Qualitative analysis

A Shimadzu Nexera ×2 system was used for qualitative analysis; it was equipped with a photodiode array detector SPD-M20A in series to a Shimadzu LCMS-8040 triple quadrupole system with an electrospray ionization (ESI) source (Shimadzu, Dusseldorf Germany). An Ascentis Express RP-Amide column (10 cm × 2.1 mm, 2.7 µm, Supelco, Bellefonte, USA) was used; mobile phase A was water/formic acid (999:1, v/v) and mobile phases B was acetonitrile/formic acid (999:1, v/v), respectively. The flow rate was 0.4 mL/min, and the column temperature was 30 °C. The gradient program was as follows: 0 to 3 min 5% B, 3 to 20 min 5% to 15% B, 20 to 30 min 15% to 25% B, 30 to 42 min 25% to 75% B, 42 to 52 min 75% to 100% B, and 52 to 53 min 100% B. The total analysis time including pre- and post-running was 60 min. UV spectra were acquired from 220 to 450 nm. Mass spectrometer operative conditions were as follows: Heat block temperature, 200°C; desolvation line (DL) temperature, 230 °C; nebulizer gas (N_2) flow rate, 3 L/min; and drying gas (N_2) flow rate, 15 L/min. Full scan mass spectra were acquired from 50 to 2000 m/z both in positive and in negative scan mode, with an event time of 0.5 s. When pseudomolecular ions $[M+H]^+$ in ESI^+ or $[M-H]^-$ in ESI^- were identified, they were submitted to collision (collision energy, -35.0 V for ESI^+ and 35.0 V for ESI^-) in product ion scan mode with an event time of 0.2 s. Selected reaction monitoring acquisition on specific product ions derived from precursor ion fragmentation was performed. Retention times, UV, and MS spectra were used to identify and tentatively identify the main components of the extracts. These data were compared with those of authentic commercial standards or when not available, to the literature data (see Table 1 and paragraph 4.3.1.2).

4.2.4.2. Quantitative analysis

Each extract (5 µL) was analyzed in triplicate with a Shimadzu UFLC XR (Shimadzu, Dusseldorf, Germany) equipped with a photodiode array detector SPD-M20A using the same column, mobile phases, flow rate, and gradient program as in the qualitative analysis (see paragraph 4.2.4.1.). UV spectra were acquired in the 220–450 nm wavelength range, and the resulting chromatograms were integrated at 270 nm, to process the analysis for the offline combination of antioxidant assays and LC analysis, and at the λmax of the identified peaks (see Table 2) for quantitative analysis. Quantitation was performed by an external standard calibration method, and the results expressed as mg of compound per g of matrix (mg/g). The calibration curves of caftaric acid and quercetin 3-*O*-glucuronide were built up by analyzing them at five concentrations in methanol/water (30:70 v/v) in the range 100–1000 mg/L, while for rutin, quercetin 3-*O*-glucoside, and kaempferol 3-*O*-glucoside, seven concentrations in the range 5–250 mg/L were used. Quantitation was performed

on eight target compounds, using the calibration curves built up on the same compound or, when not available, with those of compounds belonging to the same chemical class. The calibration curves and the analytical performances of the method are described in Table 2. The analytical performances were measured in terms of repeatability (RSD% never exceeding 5%) and intermediate precision (RSD% never exceeding 10%). All data were processed using LabSolution software (Shimadzu, Dusseldorf Germany).

Table 2. Wavelengths, calibration ranges, equations of the curves and linearity of the target compounds used for quantification.

Compound	λ max (nm)	Linearity range (mg/l)	R^2	Calibration curve equation
Caftaric acid	325	100-1000	0.9994	y=21869x-260461
Quercetin 3-*O*-glucuronide	350	100-1000	0.9992	y=14009x-235766
Rutin	350	5-250	0.9972	y=13724x-5740.1
Quercetin 3-*O*-glucoside	350	5-250	0.9979	y=16561x-9313.8
Kaempferol 3-*O*-glucoside	350	5-250	0.9979	y=3864x-3225.3

4.2.5. Total phenolic content assay

The total phenolic content was determined as described by Singleton and Rossi [46] with slight modifications. First, 250 µL of the extracts (diluted 1:25 in methanol) were added to 4 mL of water, together with 250 µL of pure Folin–Ciocalteu's phenol reagent, and 500 µL of an aqueous solution of Na_2CO_3 (pH = 10). Then, the absorbance was measured at 765 nm after one hour with a UV/Visible spectrophotometer (Genesys 6, Thermo Electron Co., Madison, WI, USA). The results were expressed as mg of gallic acid equivalent (GAE) per g of matrix. The calibration curve of gallic acid was built up with the same method, by analyzing its standard at concentrations ranging from 0.01–0.5 mg/mL.

4.2.6. Antioxidant activity determination

4.2.6.1. Scavenging effect on DPPH• radicals

The capacity to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) was monitored with the method reported by Król et al. [31] with some modifications. The extract solution (10 µL of the extract diluted 1:5 in methanol) was mixed with 2 mL of a methanol solution containing DPPH• radical (30 µL/mL). The mixture was shaken vigorously and left to stand for 30 min at room temperature in the dark (until absorbance values were stable). Reduction of the DPPH• radical was measured by monitoring the absorption decrease at 515 nm. The DPPH scavenging effect was calculated with the following equation:

$$\% \text{ scavenging effect} = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$$

where A_{DPPH} is the absorbance of the DPPH• solution and A_S is the absorbance of the DPPH• solution after addition of the sample extract. The amount of matrix used to prepare an extract providing 50% inhibition (EC_{50}) was extrapolated from the % scavenging effect.

4.2.6.2. Scavenging effect on ABTS$^{+\bullet}$ radicals

The ABTS method was applied as per Król et al. [31] with slight modifications. The ABTS radical was generated by chemical reaction with potassium persulfate ($K_2S_2O_8$). First, 5 mL of $K_2S_2O_8$ solution (0.66 mg/mL) were added to 5 mL of ABTS (3.84 mg/mL), then, the solution was kept in the dark for 12 to 16 h, at −20 °C, to form the radical. An accurate volume of the previous solution was diluted in ethanol/water (50:50 v/v) until absorbance of 0.70 ± 0.02 at λ = 734 nm was achieved. Once the radical was formed, 2 mL of ABTS$^{+\bullet}$ radical solution was mixed with 100 µL of the extracts diluted 1:100 in ethanol, and the absorbance at λ = 734 nm measured after 6 min. The ABTS$^{+\bullet}$ scavenging effect was calculated as equivalent mmol of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) per g of matrix. The Trolox calibration curve was built up by analyzing the standard compound at concentrations ranging from 0.025 to 0.3 mM, with the same method.

4.2.6.3. Off-line combination of antioxidant assays and HPLC-PDA analysis

4.2.6.3.1. DPPH method

First, 50 µL of each extract were added to 10 mL of DPPH$^{\bullet}$ working solution, and left to stand for 30 min at room temperature in the dark; after solvent evaporation under a gentle nitrogen stream, the residue was diluted to 500 µL with methanol and filtered through a 0.22 µL PTFE filter. 5 µL of the resulting solution were submitted to LC analysis. The chromatographic pattern of the extract after reaction with DPPH$^{\bullet}$ radical solution was compared with that of the same extract before reaction, diluted 1:10.

4.2.6.3.2. ABTS method

First, 50 µL of extract were added to 10 mL of ABTS$^{+\bullet}$ working solution, and left to stand for 6 min at room temperature in the dark, then after evaporation under a gentle nitrogen stream the residue was diluted to a volume of 1 mL with methanol, and filtered through a 0.22 µL PTFE filter. 5 µL of the resulting solution were analyzed by LC. The chromatographic pattern of the extract after reaction with ABTS$^{+\bullet}$ radical solution was compared with that of the same extract before reaction, diluted 1:20. For both the assays, the procedure was repeated thrice with highly consistent results (RSD% never exceeding 10%) and the percent peak area reduction was calculated on the mean areas.

4.2.7. Statistical analysis

All analyses were performed in triplicate and data were expressed as mean values ± standard deviation. All statistical elaboration (experimental designs and their elaboration, principal component analysis, box plots, and Student's t-test) were carried out using Statistica 10 (StatSoft. Inc., Tulsa, OK, USA) software. The experimental design was performed in order to optimize the extraction method of the raw plant material. Principal component analysis (PCA) was conducted to verify similarities and dissimilarities among the investigated samples, and box plots and Student's t-test were used to define statistical differences concerning compound abundances and antioxidant power of the extracts ($p < 0.01$).

4.3. Results and discussion

4.3.1. Phytochemical analysis of grapevine green pruning residues (GPRs)

Grapevine phytochemical composition was studied by HPLC-PDA-MS/MS. The analyses were performed on both green pruning residues and leaves on samples from the same plants, because only information for the leaves is available from the literature [7,23,27,28]. The results were then compared to evaluate differences and similarities in the chemical composition of the two investigated samples.

4.3.1.1. Optimization of the extraction of the phenolic compounds of GPRs

The GPRs were extracted by ultrasound-assisted extraction (UAE), an easy-to-handle technique that can be run at room temperature, providing correct extraction of thermolabile compounds. Moreover, it is a green chemistry technique, entailing low solvent and energy consumption while providing a high extraction yield and fast kinetics [36,37].

The extraction method was optimized through experimental design, i.e., an approach giving the most effective combination of parameters to run the ultrasound extraction process to obtain the highest polyphenolic yield. A pool sample of GPRs, which was obtained by mixing an equal amount of GPRs from each cultivar, was used as the model sample for optimization.

The first step was to select the optimal extraction solvent. Ethanol, acetone, and a mixture of methanol/water (70:30 v/v) were chosen for the GPRs pool sample extractions, based on the literature [7]. HPLC analysis of the different extracts revealed that the three solvents gave comparable qualitative results, but methanol/water provided the highest recovery (data not shown). The main variables affecting extraction (i.e., solvent, extraction time, amount of matrix, and volume of solvent) were then screened by applying a Box, Hunter and Hunter design (see Table 3). For temperature, it is important to consider that higher temperatures can improve mass transfer during extraction, but, at the same time, it can promote a high component degradation rate, in particular above 75 °C [38]. The UAE process was, therefore, carried out at 30 °C.

The extraction efficiency of each experiment was evaluated in terms of both the UV peak area of every compound detected at 270 nm, and of the sum of the areas of all peaks. The effects of the variables on the sum of all peak areas, and the peak area of the most representative compounds, are illustrated by Pareto charts in Figures 1A and 2 (A1–A4), respectively. All variables were significant ($p < 0.05$) with an increase of the response when passing from the lowest to the highest level. The final optimization of the variables most influencing the extraction process (i.e., volume of solvent and amount of matrix) was carried out applying a central composite design (CCD) keeping extraction time (15 min) and water concentration (30%) in the water/methanol solvent mixture constant (Table 3).

Surface response plots show the relationships between extraction parameters and analyte response (extraction yield). Figures 1B and 2 (B1–B4) show the response surface plots correlating the effect of matrix amount and solvent volume to both the extraction yield of the most abundant phenols and the sum of all peaks. All surface responses clearly indicate that extraction yield improves as the

two variables increase. The mathematical model developed showed good consistency between experimental and predicted values (data not shown).

In conclusion, the optimum extraction conditions were the following: 15 min extraction time, 30% water in the solvent mixture, 500 mg matrix, and 50 mL solvent. To make the method quicker and more sustainable, extraction was carried out reducing both the amount of plant material and the solvent volume, while maintaining the ratio between them constant. Under the new conditions (i.e., 15 min extraction time, 30% water in the solvent mixture, 100 mg matrix, and 10 mL solvent), the extraction results were perfectly comparable with the previous results. Moreover, the single-step process was also exhaustive as it was confirmed by the lack of peaks detected in the chromatogram of a second extraction on the same plant material (data not shown).

Table 3. Variables, levels and the design matrices evaluated in the two experimental design.

	Independent variable	**Coded Variable**	**Levels**				
			-α	**-1**	**0**	**+1**	**+α**
Box, Hunter & Hunter Design	Water in the solvent mixture (%)	*x1*		0	25	50	
	Extraction time (min)	*x2*		10	20	30	
	Amount of matrix (mg)	*x3*		100	250	500	
	Volume of solvent (ml)	*x4*		5	22.5	50	
Central Composite Design	Amount of matrix(mg)	*x3*	138	200	350	500	562
	Volume of solvent(ml)	*x4*	13.8	20	35	50	56.2

	Box, Hunter & Hunter Design				**Central Composite Design**	
Run	*Variables*				*Variables*	
	x1	*x2*	*x3*	*x4*	*x3*	*x4*
1	-1	-1	-1	-1	-1	-1
2	1	-1	-1	1	-1	1
3	-1	1	-1	1	1	-1
4	1	1	-1	-1	1	1
5	-1	-1	1	1	-α	0
6	1	-1	1	-1	+α	0
7	-1	1	1	-1	0	-α
8	1	1	1	1	0	+α
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0	0	0	0	0

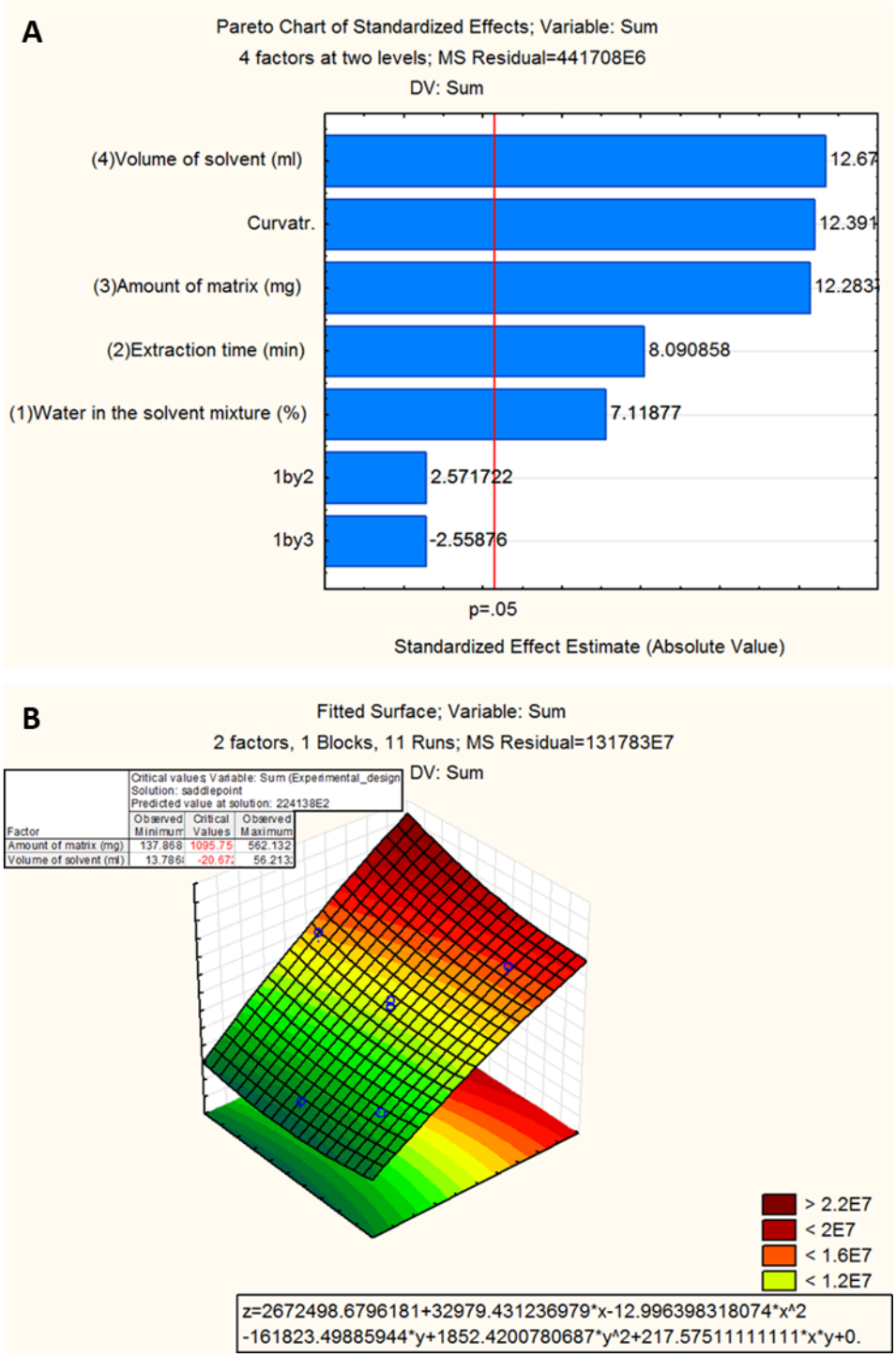


Fig. 1. Results of the optimization of extraction conditions obtained by experimental design: **A**) Pareto Chart of Effects relative to the sum of the peak areas of the HPLC-PDA chromatographic profiles of the extracts, obtained through the Box, Hunter & Hunter Design; **B**) Surface Response and Critical Values relative to the sum of the peak areas of the HPLC-PDA chromatographic profiles of the extracts, obtained through the Central Composite Design.

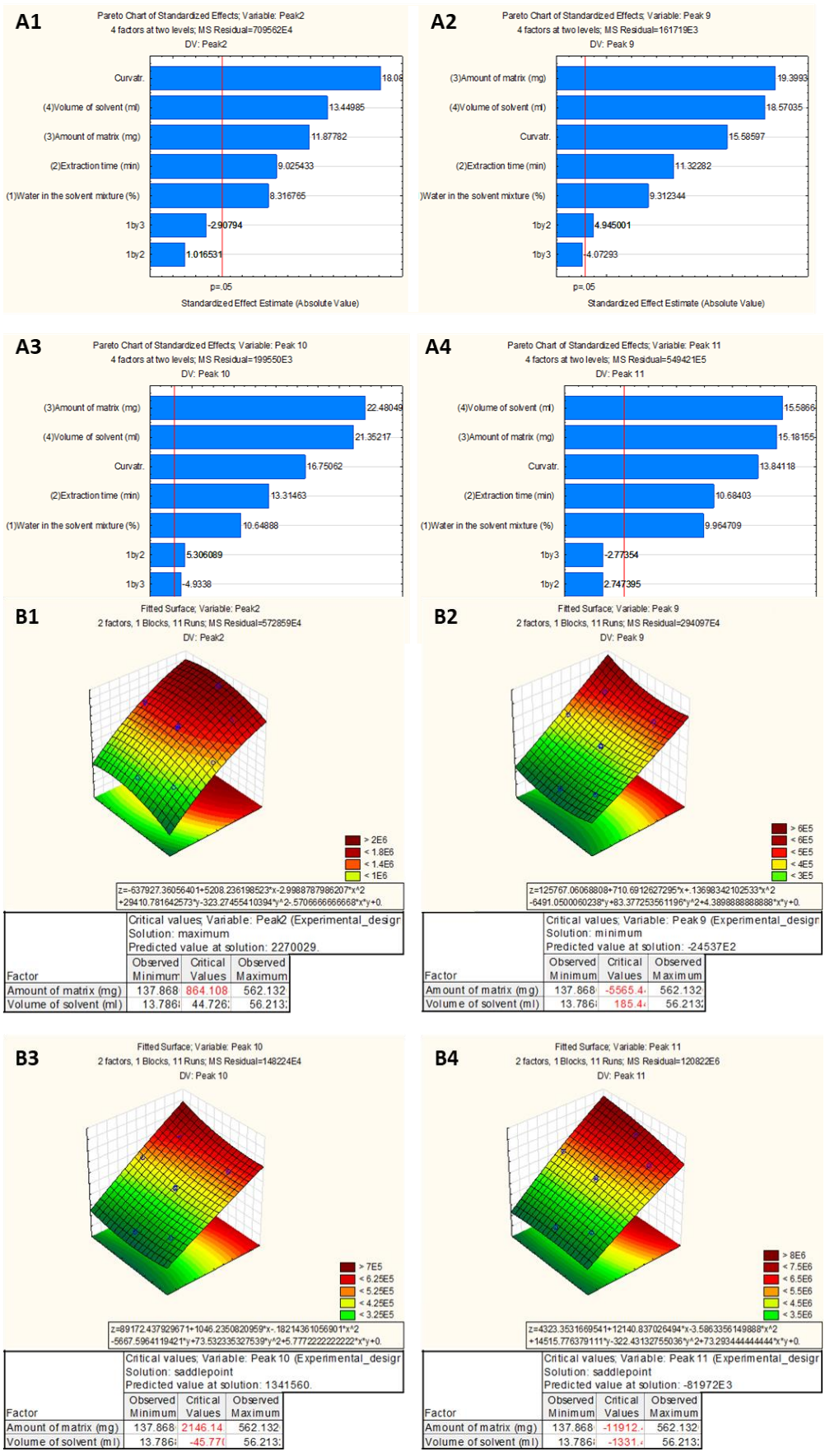


Fig. 2. Results of the optimization of the extraction conditions obtained by the experimental design. Pareto Chart of Effects relative to the main components of the extracts obtained through the Box, Hunter & Hunter Design: **A1**) peak 2, **A2**) peak 9, **A3**) peak 10, **A4**) peak 11. Surface Response and Critical Values relative to the main components of the extracts obtained through the Central Composite Design: **B1**) peak 2, **B2**) peak 9, **B3**) peak 10, **B4**) peak 11.

4.3.1.2. Identification and quantification of the phenolic compounds of GPRs

To the best of the authors' knowledge, no data are available on the phenolic composition of *V. vinifera* green pruning residues (GPRs), whereas some phytochemical investigations on grapevine leaves have been reported [7,23,27,28]. Therefore, these studies were taken as a reference, since HPLC-PDA-MS/MS patterns of GPRs and leaf hydroalcoholic extracts showed similar chemical compositions (Figure 3), with a marked abundance of phenolic compounds.

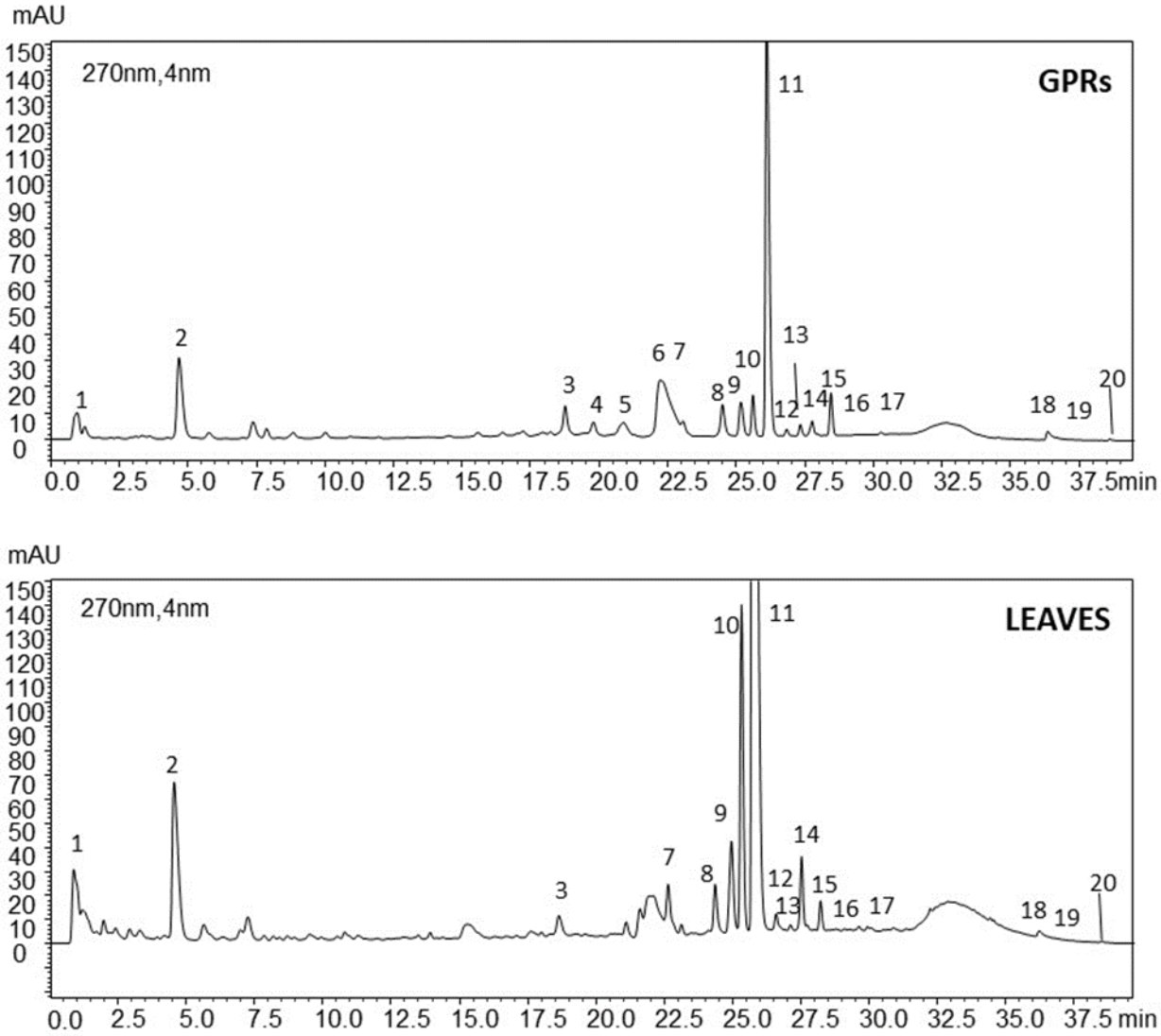


Fig. 3. HPLC-PDA profiles (λ: 270 nm) of GPRs and leaves. Peak numbers corresponding to compounds listed in Table 4.

Chromatographic patterns, UV, and mass spectral data detected 20 informative compounds. The UV spectra for each peak provided a preliminary indication of the group of compounds. The molecular weight of each peak was also defined by its mass spectral pattern, through the complementary correspondence between positive and negative pseudomolecular ions in ESI^+ and ESI^- modes. The product ion scan analysis of the pseudomolecular ions under investigation provided diagnostic fragments for each compound. The identity of 11 compounds in the extract was confirmed by co-injection of authentic commercial standards, whereas eight peaks were putatively identified by comparison with literature data (Table 4).

Table 4. List of identified and putatively identified compounds in *V. vinifera* leaf and green pruning residue (GPR) extracts. For each analyte, retention time, UV maximum (a), pseudomolecular ions, fragment ions obtained by Product Ion Scan mode (PIS) and identified or tentatively identified compound names are given. Identification Confidence values and references are also included.

N°	RT (min)	λ max (nm)	$[M+H]^+$ m/z	$[M-H]^-$ m/z	Supp. MW	M^{2+} m/z	M^{2-} m/z	Compound name[a]	Leaves	GPRs	Ident. confidence[b]	Ref.
1	1.2	277	/	331	/	/	59 71 89 123 151 169 211	Galloylglucose	X	X	2	[39]
2	4.6	326/244	/	311	312	/	/	**Caftaric acid**	X	X	1	[27, 40]
3	18.6	273	/	631	/	/	613 479 445 301 273 229	Hydrolyzable tannin	n.d.	X	3	[39, 41, 42]
4	19.6	SH 280	1431	1429	1430	321 303	753	Hydrolyzable tannin	n.d.	X	3	[41]
5	20.7	273	1431	1429	1430	1057 849 427 303	753	Hydrolyzable tannin	n.d.	X	3	[41]
6	22.1	SH 280	/	861 815 779	/	/	751 301 273	Hydrolyzable tannin	n.d.	X	3	[41, 42]
7	22.9	255/348	495	493	494	319	317	Myricetin glucuronide	X	X	2	[23]
8	24.3	275	803	801	802	153 337 633	765	Vitilagin or isovitilagin	X	X	3	[43]
9	25.1	255/356	611	609	610	303	301	**Rutin**	X	X	1	[23, 28,40]
		254/353	465	463	464	303	301	**Hyperoside**	X	X	1	[23, 40]
10	25.5	254/352	465	463	464	303	301	**Quercetin 3-*O*-glucoside**	X	X	1	[23, 40]
11	26	255/352	479	477	478	303	301	**Quercetin 3-*O*-glucuronide**	X	X	1	[23, 40]

12	26.7	266/350	595	593	594	287	285	**Kaempferol 3-*O*-rutinoside**	X	X	1	
13	27.2	271/353	625	623	624	317	315	Isorhamnetin *O*-dihexoside (glucose + rhamnose)	X	X	2	
		271/353	479	477	478	317	315	Isorhamnetin hexoside	X	X	2	
14	27.5	264/349	449	447	448	287	285	**Kaempferol 3-*O*-glucoside**	X	X	1	[23, 27, 40]
		264/349	551	549	550	303	301	Quercetin malonylhexoside	X	X	2	
15	28.3	272/352	493	491	492	317	315	Isorhamnetin glucuronide	X	X	2	
16	29.5	275/353	535	533	534	287	285	Kaempferol malonylhexoside	X	X	2	
17	30.2	275/353	565	563	564	317	315	Isorhamnetin malonylhexoside	X	X	2	
		306	229	227	228	/	/	**Resveratrol**	traces	n.d.	1	[28]
18	36.4	254/368	303	301	302	/	/	**Quercetin**	X	X	1	[23, 28]
19	37.5	254/368	317	315	316	/	/	**Isorhamnetin**	X	X	1	[23]
20	38.5	265/366	287	285	286	/	/	**Kaempferol**	X	X	1	[23, 28]

[a] In bold, compounds identified by comparison with reference standards.

[b] Identification confidence as stipulated by the CAWG [46]: Level 1: Identified Compound (a minimum of two independent and orthogonal data, such as retention time and mass spectrum) compared directly relative to an authentic reference standard; Level 2: Putatively Annotated Compound (compound identified by analysis of spectral data and/or similarity to data in a public database); Level 3: Putatively characterized Compound Class Level.

In particular, compounds for which authentic commercial reference standards were not available were tentatively identified through their tandem mass spectrometry fragmentation pattern, as reported by Marengo et al. [44]. This approach provides further structural information on unknown compounds. For example, vitilagin (or isovitilagin) was tentatively identified by its UV spectral data (UVmax= 275 nm), pseudomolecular ions 803 m/z and 801 m/z, in ESI^+ and ESI^- Ionization modes, respectively, and fragmented to give diagnostic ions at m/z 153, 337, and 633 in ESI^+ and m/z 765 in ESI^-[43]. Other hydrolyzable tannins were putatively hypothesized on the basis of their UV maximum spectral absorption and diagnostic fragments at m/z 753, 301, 273, and 229 [39,41,42]. Selected reaction monitoring acquisition (SRM) was also carried out on specific product ions, providing further structural information on the investigated compounds. In agreement with data reported for polyphenols of *V. vinifera* leaves, flavonoids were the most representative group in GPRs hydroalcoholic extracts, in particular in the form of O-glycosides of quercetin, kaempferol, myricetin, and rhamnetin (or isorhamnetin). A phenolic acid derivative (i.e., caftaric acid) and some hydrolyzable tannins (including vitilagin and isovitilagin) were also detected [23,40,43]. Interestingly, GPRs extracts did not contain stilbenes, although traces of resveratrol were detected in leaves [28].

Figure 4 shows the quantitative results on GPRs and leaves highlighting the quantity of each target compound in relation to the total amount of phenolic compounds quantified. It is noticeable that the total content of phenolics, as well as their proportions, are similar in GPRs and in leaves, quercetin 3-*O*-glucuronide, caftaric acid, and quercetin 3-*O*-glucoside being the most abundant compounds in both matrixes. The total phenolic content determined by the Folin–Ciocalteu method is in accordance with HPLC quantification. The concentration of total phenolics was expressed as mg of gallic acid equivalent (GAE) per g of sample and no statistical differences emerged among GPRs and leaves (Figure 5).

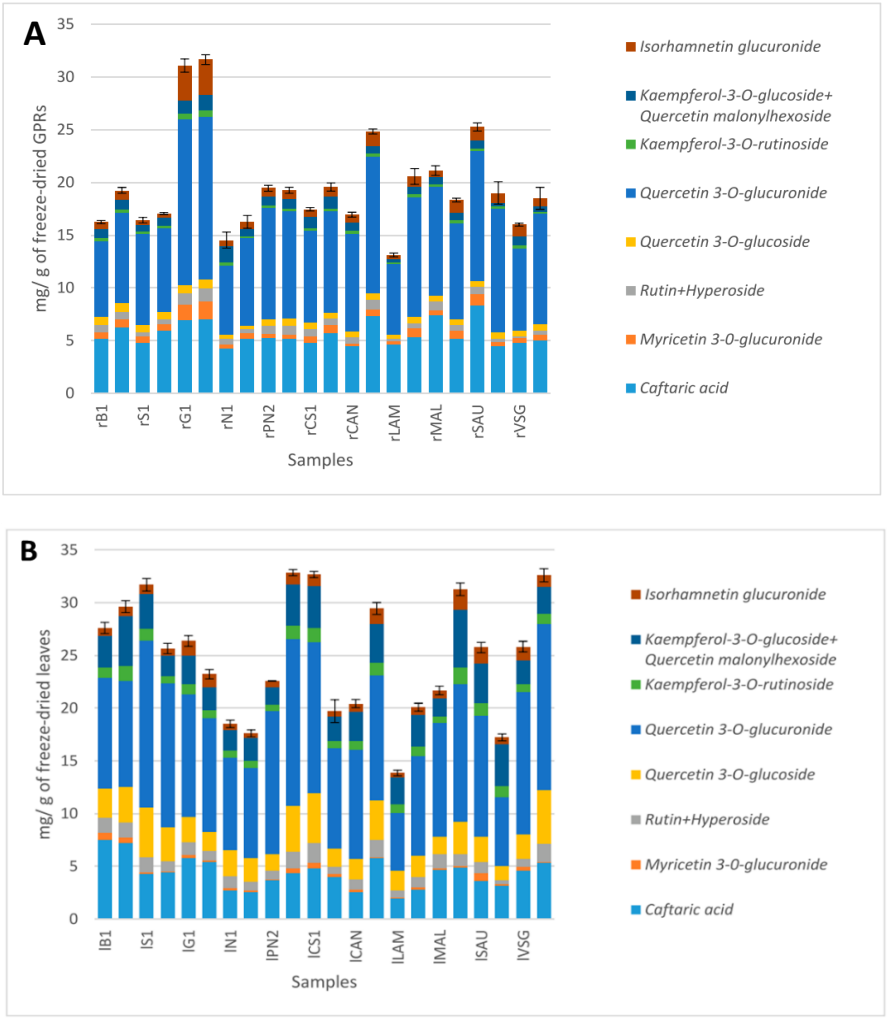


Fig. 4. Concentration (mg/g) of the main phenolic compounds of freeze-dried green pruning residues (GPRs) (**A**) and leaves (**B**).

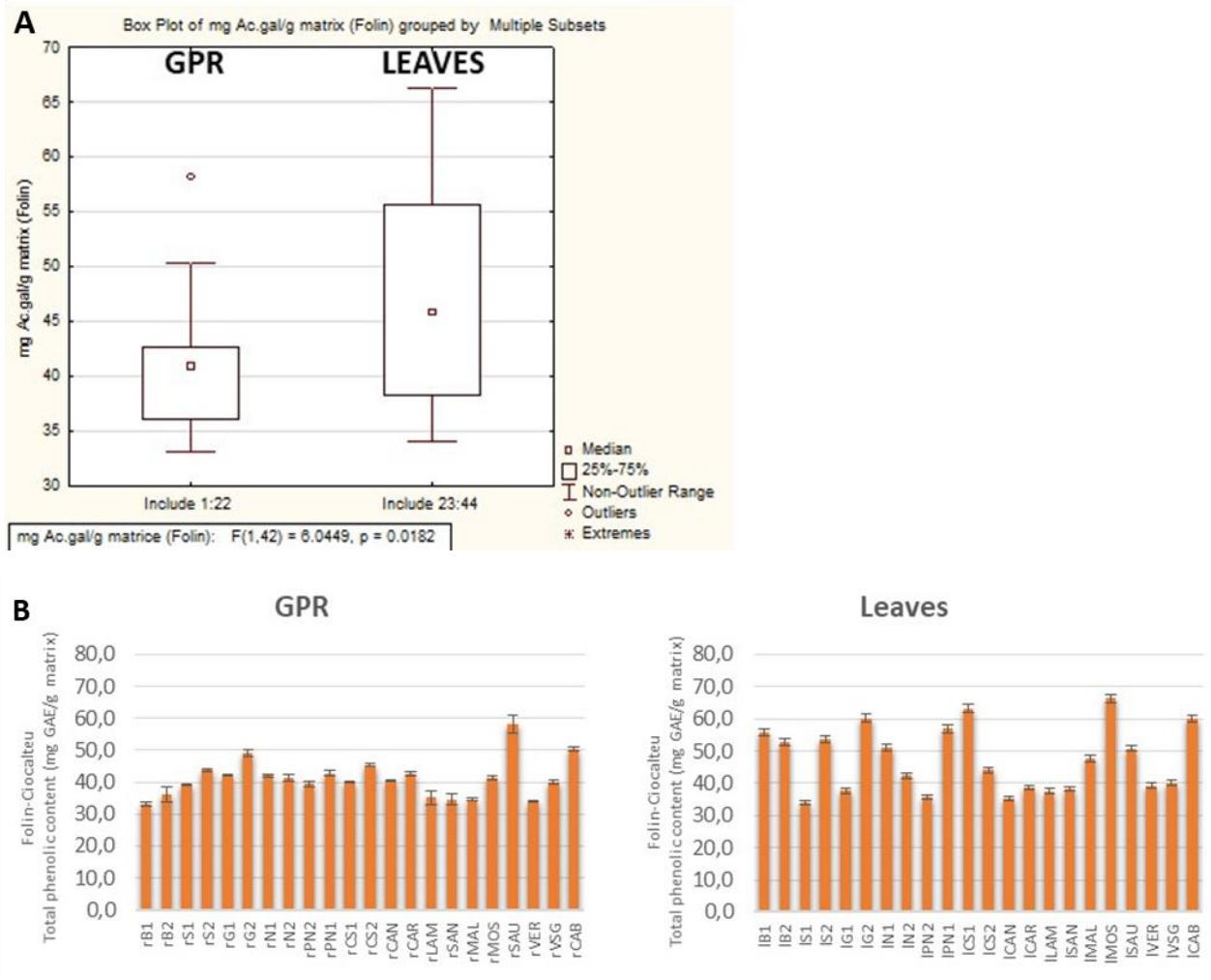


Fig. 5. Box Plots (**A**) and histograms (**B**) relative to the Folin–Ciocalteu Assay. Total phenolic content is expressed as mg GAE/g matrix for both the GPRs and leaf extracts.

4.3.1.3. Statistical analysis and comparison of the phytochemical patterns of GPRs and leaves

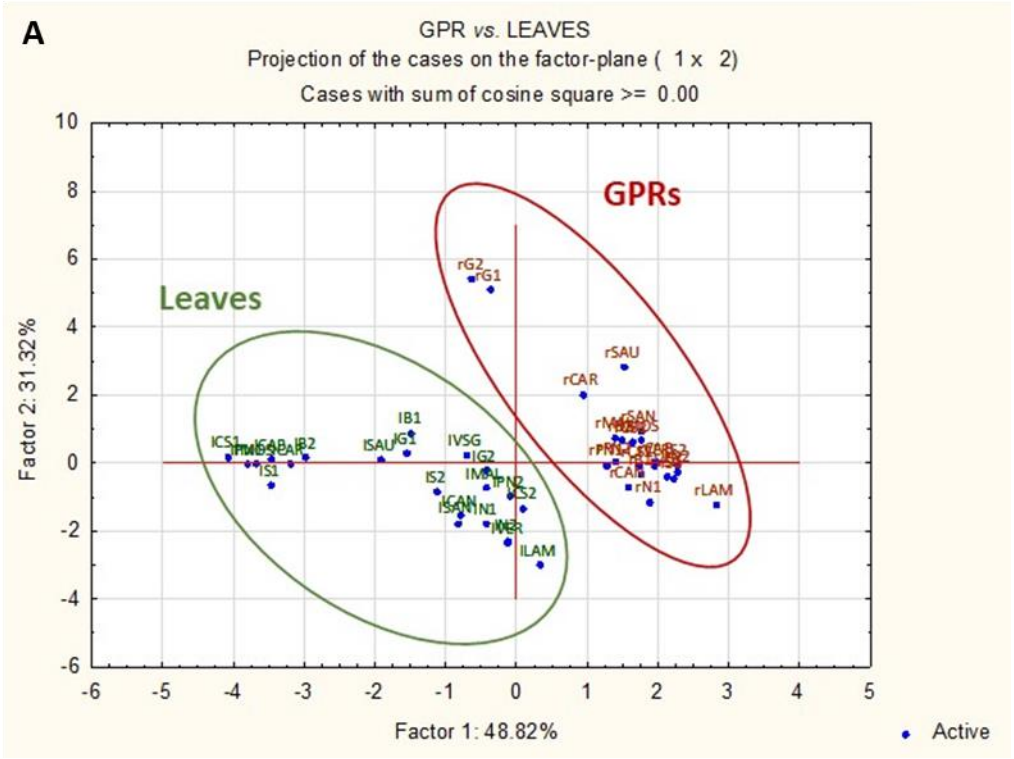
Since *V. vinifera* GPRs and leaves showed very similar polyphenolic profiles, principal component analysis (PCA), an unsupervised multivariate data analysis method, was applied to evaluate the possibility of discriminating between the two matrices, using as variables, the eight quantified components.

The scores and the loading plots are shown in Figure 6. The first (Factor 1) and second (Factor 2) components (48.82% and 31.32% of explained variation, respectively) clearly discriminate between GPR and leaf samples. A Student t-test was, therefore, applied to each variable;

Figure 7 shows the box plots for the quantified compounds to compare their abundance in GPRs and leaf samples. The box plots show that caftaric acid and myricetin glucuronide are more abundant in GPR extracts ($p < 0.01$) and positively correlated with the second principal component (Figure 6B). Conversely, rutin and hyperoside, quercetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, and kaempferol 3-*O*-glucoside and quercetin malonyl hexoside are more abundant in leaf extracts ($p < 0.01$) and negatively correlated with the first principal component (Figure 6B). The amounts of

quercetin 3-*O*-glucuronide and isorhamnetin glucuronide in GPRs and leaves ($p > 0.01$) were not significantly different.

These results indicate that, although *V. vinifera* GPRs and leaves have similar chemical patterns, there are some differences in the relative abundances of common components.



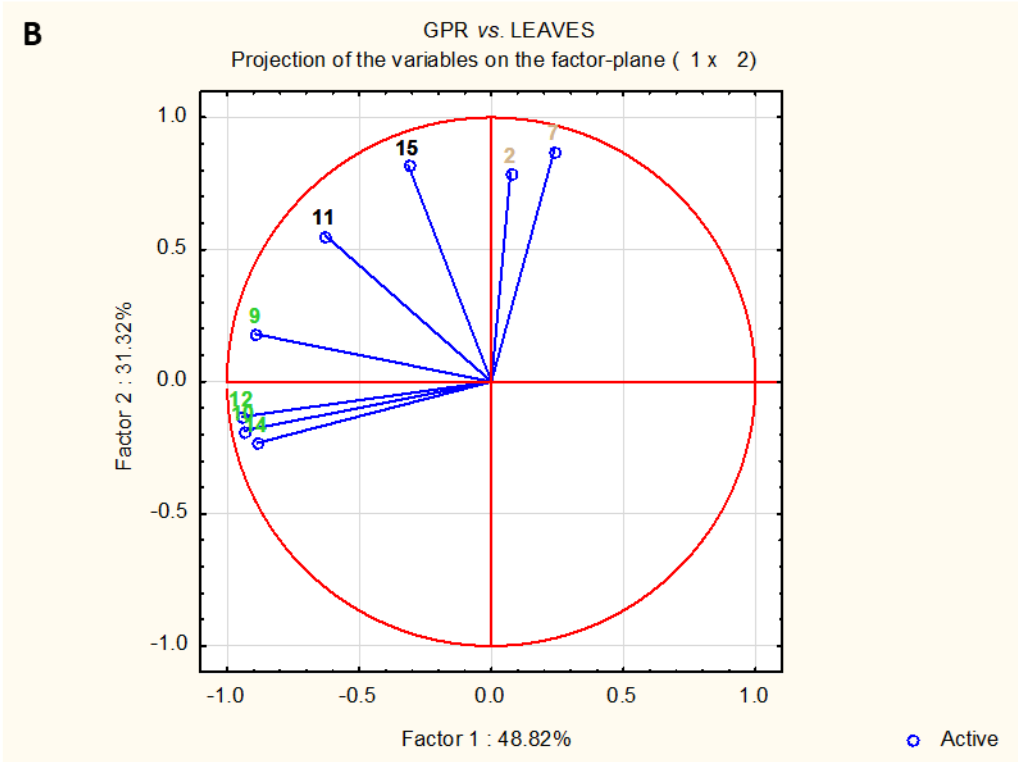


Fig. 6. Score plot (**A**) and loading plot (**B**) of the Principal Component Analysis relative to the quantity of the main phenolic compounds in green pruning residues (GPRs) (r) and leaves (l). Legend: score plot, GPRs in brown and leaves in green; loading plot: 2) caftaric acid, 7) myricetin glucuronide, 9) rutin and hyperoside,

10) quercetin 3-*O*-glucoside, 11) quercetin 3-*O*-glucuronide, 12) kaempferol 3-*O*-rutinoside, 14) kaempferol 3-*O*-glucoside and quercetin malonylhexoside, 15) isorhamnetin glucuronide; the analytes significantly more abundant in GPRs are in brown, those significantly more abundant in leaves are in green.

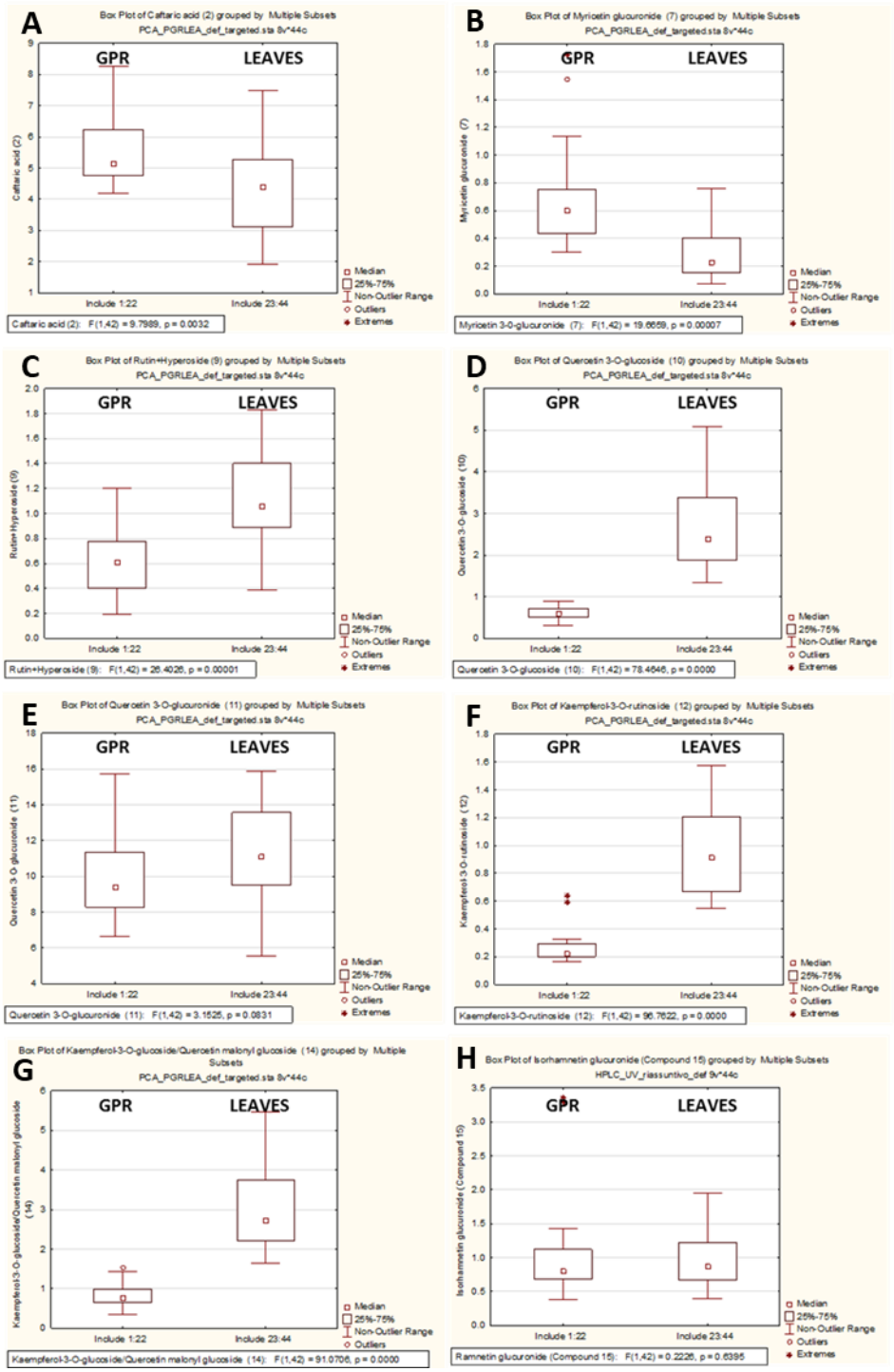


Fig. 7. Box Plots relative to the HPLC-PDA quantitative results on the main components of grapevine GPRs and leaves: **A**) caftaric acid, **B**) myricetin glucuronide, **C**) rutin and hyperoside, **D**) quercetin 3-*O*-glucoside, **E**) quercetin 3-*O*-glucuronide, **F**) kaempferol 3-*O*-rutinoside, **G**) kaempferol 3-*O*-glucoside and quercetin malonyl hexoside and **H**) isorhamnetin glucuronide.

4.3.2. Evaluation of the antioxidant potential of GPRs

4.3.2.1. In-vitro antioxidant assays (scavenging of DPPH• and ABTS+• radicals)

In a second step, the antioxidant properties of the extracts were investigated through colorimetric *in vitro* assays and the results were correlated to those obtained by HPLC. In particular, the antiradical activity was measured by evaluating the scavenging effects on the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and the 2,2'-azinobis-3-ethyl-benzthiazoline-6-sulphonate ($ABTS^{+\bullet}$) radicals. The scavenging effect on $ABTS^{+\bullet}$ radicals was expressed in terms of Trolox equivalent antioxidant capacity (TEAC), and the scavenging effect on DPPH• radicals as EC_{50}. The results are summarized in Figures 8 and 9. The leaves' antioxidant activity is in close agreement with reported data [30] and no significant differences emerged between GPRs and leaves ($p > 0.01$ for all antioxidant assays, Figure 8), although all assays showed that the leaf extracts appear more variable than that of the GPR samples. These results indicate that GPRs can be assumed to be a source of antioxidant compounds equivalent to already exploited grapevine leaves.

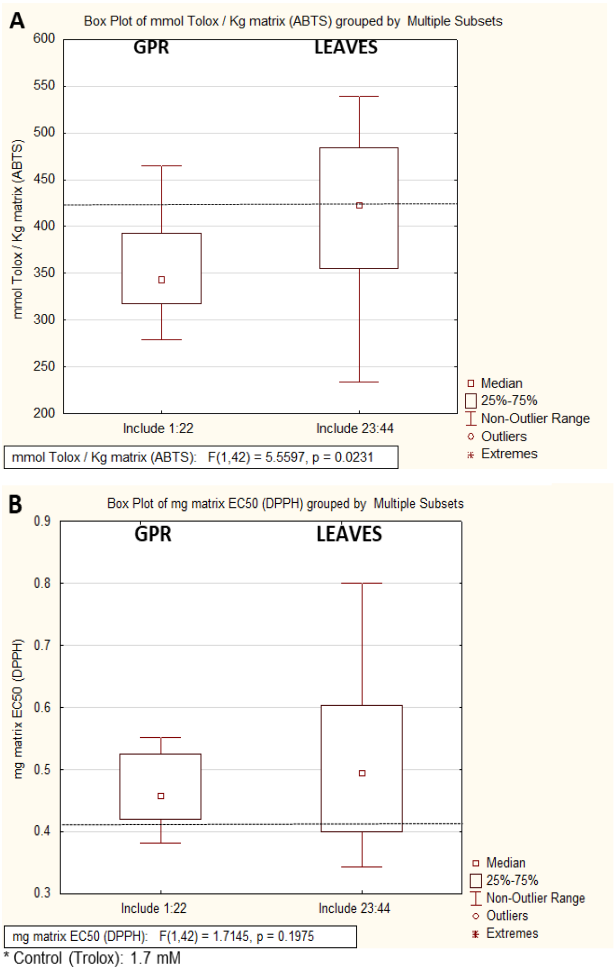


Fig. 8. Box plots relative to the in vitro colorimetric antioxidant assays: Trolox equivalent antioxidant capacity (TEAC) by ABTS Assay expressed as mmol Trolox/kg matrix (**A**) and EC_{50} (mg matrix) by DPPH assay (**B**). The dashed line gives the literature values (i.e., [30] for **A**, and [31] for **B**).

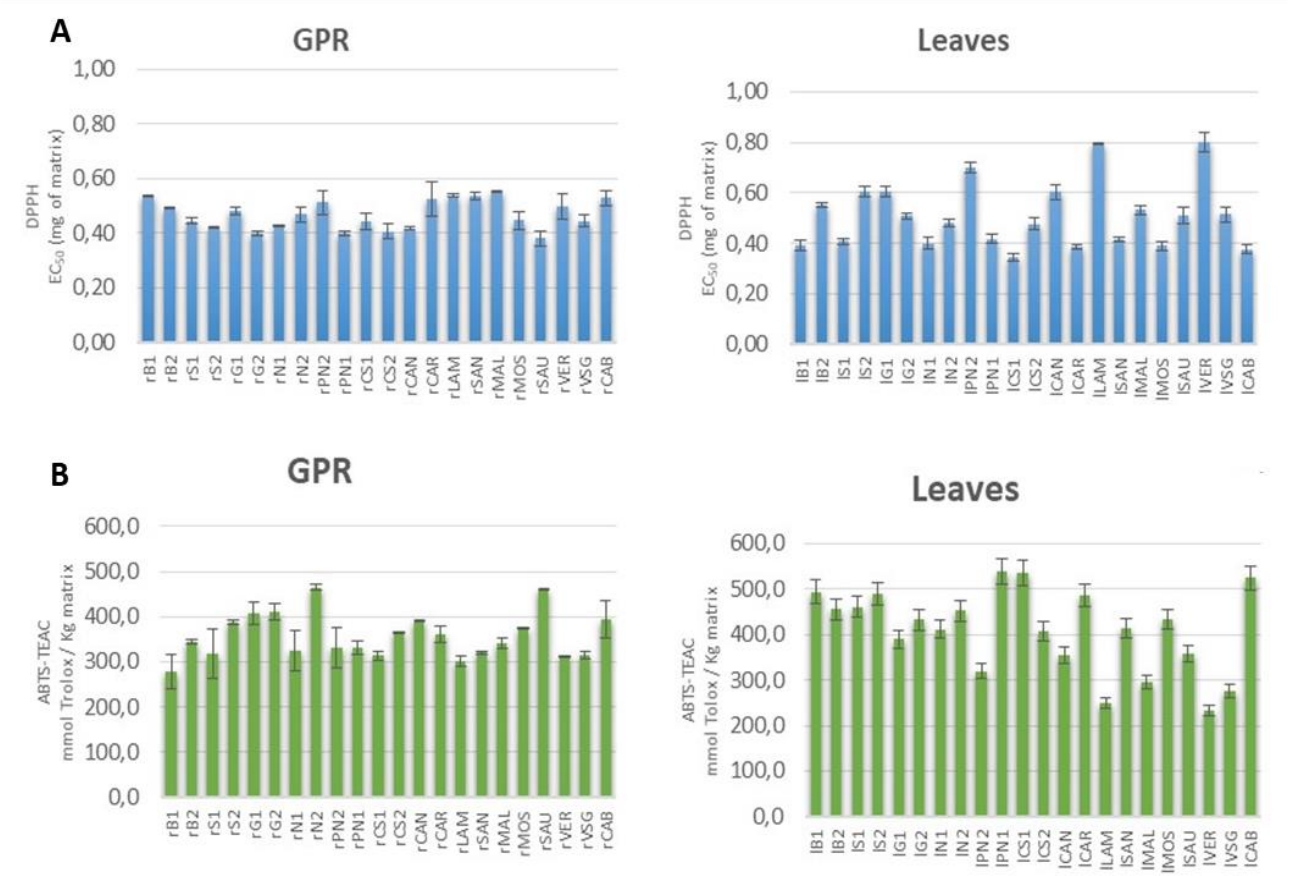


Fig. 9. Histograms relative to the *in vitro* colorimetric antioxidant assays for all GPRs and leaf extracts: EC_{50} (mg matrix) by DPPH Assay (A) and TEAC by ABTS assay expressed as mmol Trolox/kg matrix (B).

The possible correlation between the two antioxidant assays was also investigated and compared with HPLC quantitation data and the total phenols assay. Table 5 shows the Pearson correlation coefficients between the different measurements. The results with the four test techniques are consistent; their correlation is statistically different from zero, with a significance level $\alpha = 0.05$ with HPLC quantitative data, total phenolic content and TEAC correlated positively, and EC_{50} values on DPPH• correlated negatively to the other results.

Table 5. Pearson's correlation matrix between HPLC-DAD quantitation results (in terms of sum of the concentrations of the main phenolics) and *in-vitro* colorimetric antioxidant assays results.

Correlation matrix (Pearson (n-1))

Variable	Folin	ABTS	EC_{50} (DPPH)	HPLC
Folin	**1**	**0.6526**	**-0.4054**	**0.5612**
ABTS	**0.6526**	**1**	**-0.5701**	**0.6696**
EC_{50} (DPPH)	**-0.4054**	**-0.5701**	**1**	**-0.3257**
HPLC	**0.5612**	**0.6696**	**-0.3257**	**1**

Values in bold type differ from 0 at a significance level α=0.05

4.3.2.2. Off-line combination of antioxidant assays with HPLC-PDA analysis

The objective of this part of the study was to establish an efficient method for quickly identifying antioxidant active components in the phenolic extracts. Therefore, a further investigation was run to screen the component(s) of the extract(s) that mainly contributed to the antioxidant properties, in terms of their radical scavenging ability [35]. This information was obtained by analyzing with the HPLC-PDA system, the samples preliminarily submitted to the *in vitro* radical treatment. The HPLC profiles of GPR and leaf extracts, before and after reaction with DPPH$^{\bullet}$ and ABTS$^{+\bullet}$ radicals, were compared to determine the radical-scavenging activities of each phenolic component, via the percent reduction of their peak areas.

Figure 10 shows the comparison of the GPRs profiles, before and after reaction with ABTS$^{+\bullet}$ (Figure 10A) and DPPH$^{\bullet}$ (Figure 10B), and percent peak area reduction of the analytes of GPRs and leaves, after reaction with the two radicals, are listed in Table 6.

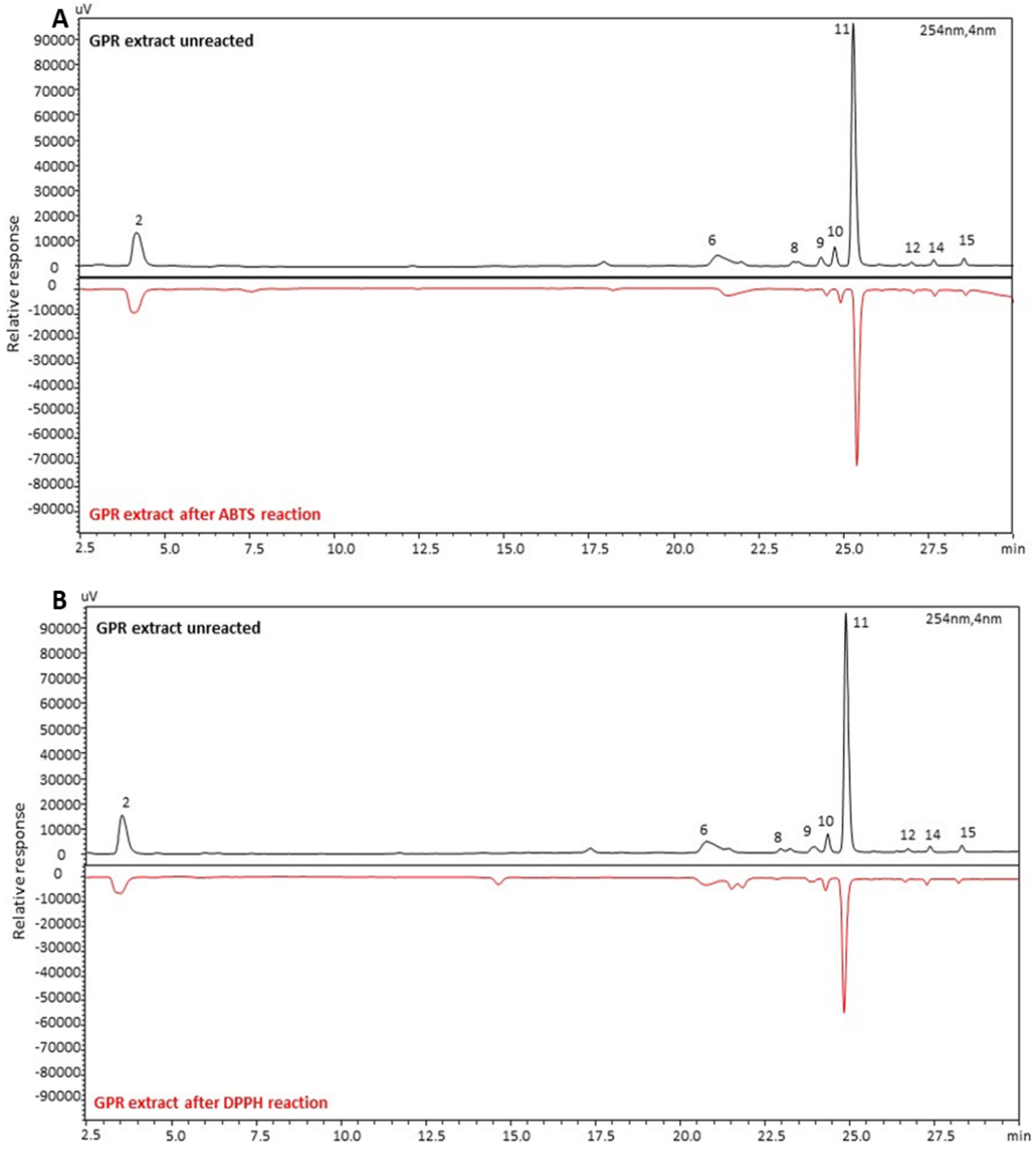


Fig. 10. HPLC chromatograms of GPRs extracts with ABTS (A) and DPPH (B) free radical before (black profile) and after (red profile) reaction. Peak numbers corresponding to compounds listed in Table 4.

Table 6. Percentage of peak area reduction of nine markers of green pruning residues (GPRs) and leaves after the reaction with the $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ radicals (n.d. = not detected). For the compound number see Table 4.

	Peak reduction (%)			
	DPPH		ABTS	
Compound N°	GPR	LEAVES	GPR	LEAVES
2	38	33	10	31
6	33	n.d.	23	n.d.
8	72	77	64	84
9	30	30	31	42
10	33	29	30	41
11	44	21	29	40
12	<1	<1	8	36
14	<1	<1	6	12
15	<1	<1	27	17

As expected, the results obtained with the two groups of samples were consistent, while some differences in scavenging activities were found between compounds and within the two radicals. In particular, compound **8** shows a very high scavenging activity (with about 70% reduction) with both $DPPH^{\bullet}$ and $ABTS^{+\bullet}$. The % reductions of caftaric acid and compound **6** for both assays were around 30%. Conversely, the different scavenging activity of the flavonoids is noteworthy, in particular against $DPPH^{\bullet}$. All quercetin glycosides show a percent reduction around 30% with both $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ radical, while kaempferol and rhamnetin glycosides show lower reduction with $ABTS^{+\bullet}$ and lower again with $DPPH^{\bullet}$. This is probably because quercetin has an additional free hydroxyl group as compared with the other aglycones. These results are in agreement with those of Zhao et al. [35].

4.4. Conclusions

This study reports the first phytochemical investigation on the polyphenolic pattern of *V. vinifera* green pruning residues (GPRs) by-products, generated by the annual pruning of vineyards in spring. The polyphenols were extracted under optimized experimental conditions, determined by experimental design, and identified and quantified, respectively, by HPLC-PDA-ESI-MS/MS and HPLC-PDA. Polyphenolics, principally consisting of flavonoids and phenolic acid derivatives, were the most interesting fraction. The phenolic phytochemical pattern of GPRs was compared with that of vine leaves and showed an equivalent qualitative composition with some quantitative differences among the components.

The antioxidant potential of GPR extracts was measured through colorimetric *in vitro* assays (scavenging of the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and the 2,2'-azinobis-3-ethyl-benzthiazoline-6-sulphonate ($ABTS^{+\bullet}$) radicals). The results were compared with those obtained for the leaf extracts and, then, with the results of HPLC investigation and total phenolic content assay, showing equivalent antioxidant properties for the two matrices and good consistency between the two techniques.

Taken together, the results suggest that grapevine GPRs are a potential source of natural compounds with valuable antioxidant properties that could be of interest in the pharmaceutical and food fields. Valorization of these by-products could, in the future, have an important economic impact, because of their low cost and ready availability. At the same time, their re-use could also

have a positive effect on the environment, as an aid to solving the problem of waste disposal associated with viticulture.

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GENERAL CONCLUSIONS

Plants has been used for centuries by mankind to prevent and treat human illnesses and the different plant parts are considered useful for several medicinal purposes. The multidisciplinary approach adopted in this Ph.D thesis in order to investigate unexploited parts of food plants with potential healthy properties: pomegranate (*Punica granatum* L.) and grapevine (*Vitis vinifera* L.), leads to the following conclusions.

Pomegranate and grapevine have been used in traditional medicine, and nowadays their specialized metabolites are widely studied to find application in pharmaceutical, food and cosmetic industry. [**Chapter 1**].

The phytochemical investigation devoted to the specialized metabolites and based on HPLC-PDA-MS/MS analysis on pomegranate bark and peel ethanolic extracts, revealed a substantial amount of tannin components while the leaf extract is characterized by the presence of flavonoids and ellagic acid as main components. Chemical and biomolecular analysis through the amplification and sequencing of some DNA barcoding genes (*ITS*, *psbA-trnH*), were carried out on different pomegranate leaf samples belonging to different sites, season and year of harvesting. The statistical data treatment of the phytochemical data through Principal Component Analysis (PCA) highligths a phenotypic variation probably related to the environment while the biomolecular analysis confirms the intra-species stability and the inter-species variability [**Chapter 2**].

Pomegranate leaf crude extract, fractions and pure compounds were then tested in order to evaluate their antiviral and anti-tumor potential. Biological *in vitro* assays were performed on two viral targets: Human immunodeficiency virus (HIV-1) and Zika virus (ZIKV), and on hematopoietic cancer cells: acute lymphoblastic leukaemia (ALL) and human multiple myeloma (MM).
Bio-guided fractionation allowed to isolate ellagic acid, the most promising compound in pomegranate leaf, which strongly inhibits both HIV-1 retro transcriptase (RT) and integrase (IN). Due to its capability to interact with two enzymatic targets, it represents a promising anti-HIV-1 agent. In the case of ZIKV, ellagic acid is not active directly on the viral particle but inhibits the first step of the viral replicative cycle which take place before the virus attachment and entry. This represents an interesting feature suggesting additional studies to evaluate its antiviral potential in *in vivo* experiments, especially for its use in prophylaxis.
In preliminary *in vitro* assays on ALL cells only ursolic acid showed the highest cytotoxic effect comparable with conventional chemotherapeutics used as controls. The studies were subsequently carried out on MM cell lines where ursolic acid showed citotoxicity and induced cell cycle arrest in G2/M phase and apoptosis in RPMI8226 cells not affecting normal lymphocytes control. These findings are confirmed by the current literature suggesting a potential therapeutic activity of ursolic acid for the treatment of multidrug resistant tumors.
In conclusion, pomegranate leaf extract deserves further investigations being an interesting source of bioactive molecules which may became novel therapeutic drugs [**Chapter 3**].

The polyphenolic pattern of grapevine green pruning residues (GPRs) generated by the annual pruning of vineyards in spring was extracted under optimized experimental conditions and evaluated by HPLC-PDA-ESI-MS/MS analyses. The comparison with the polyphenolic composition of the vine leaves, showed an equivalent profile. In addition, colorimetric *in vitro* assays ($DPPH^{\bullet}$ and $ABTS^{+\bullet}$) evaluate the antioxidant potential of GPR extracts, showed equivalent antioxidant

properties for the two matrices. On the basis of these results, GPRs represent a potential source of natural compounds with antioxidant properties for human health and their valorization in the future can increase their economic value reducing the environmental impact as a waste by-product [**Chapter 4**].

In conclusion, this Ph.D. thesis shows the usefulness of a multidisciplinary approach to investigate poorly known unexploited parts of food plants, which can be a promising source of bioactive compounds. Further studies have yet to be done to support the traditional medical uses of these plants, promoting the development of food supplements and herbal medicinal products.

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Nec aspera terrent