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Study of medicinal and food plants as a source of biologically active compounds.

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Abstract

In the last few decades, scientific evidence has pointed out the health-beneficial effects of phenolic compounds present in fruits, vegetables, and grains, and a high intake of these foods has been linked to a lower risk of most common degenerative and chronic diseases that are known to be caused by oxidative stress. In this frame can be inserted research carried out during my PhD thesis, which concerns the phytochemical investigation of phenolic composition in fruits and seeds of food plants, in particular **sweet cherry** (Prunus avium L.), **apple** (Malus domestica L.) fruits and **quinoa** (*Chenopodium quinoa* Willd.) seeds.

Sweet cherries are highly appreciated fruits for their taste, color, nutritional value, and beneficial health effects. Despite the high number of cultivars present in Italy, there is considerable interest in obtaining new ones that would extend the harvesting season. Among the most desirable fruit quality traits, there are very early and/or late ripening time, large cherry size, light or dark red color, firmness, sweetness, and taste. Several breeding programs have been conceived with the aim of releasing on the market new cultivars, possessing these kinds of improved quality attributes. For this purpose, more than thirty years ago the University of Bologna launched a sweet cherry breeding program, which allowed to release seven new cultivars. The first project of my PhD thesis was focused on the investigation of phytochemical profile and nutraceutical value of these new sweet cherry cultivars. The profile of three classes of phenolic compounds and their antioxidant activity were investigated in extracts prepared from sweet cherry fruits through high-performance liquid chromatography with diode array detection (HPLC-DAD) and spectrophotometric assays, respectively, and compared with those of commonly commercialized cultivars. The nutraceutical value of the new cultivars was investigated in terms of antioxidant/neuroprotective capacity in neuron-like SH-SY5Y cells, on order to investigate their ability to counteract the oxidative stress and/or neurodegeneration process.

The second project on food plants was focused on the phytochemical analysis of secondary metabolites in ancient cultivars of apple (*Malus domestica* L.). Although more than 7500 varieties of apples exist worldwide, during the past few decades many heritage varieties have been abandoned, in favor of mainstream varieties derived from intensive

selective breeding programs. The reason for this is the consumers' demand for sweet and crisp apples which are uniform in size and appearance and have greater disease resistance and prolonged shelf life. Despite that, in recent years some ancient varieties have been re-evaluated, mainly thanks to the health-promoting effects that have been recognized for apple phenolic compounds, including a preventive activity towards some types of cancer. To this purpose, the research work consisted of the characterization of 35 ancient cultivars of apple for their composition in phenolic compounds, in comparison with two commercial cultivars. The aim of this project was to select the most diverse cultivars, that will then be assayed for their anti-carcinogenic and anti-proliferative activities against the hepato-biliary and pancreatic tumors.

The third project on food plants was focused on the analysis of polyphenolic pattern of seeds of quinoa (*Chenopodium quinoa* Willd.). The consumption of these seeds ensures a valuable intake of macronutrients, micronutrients, minerals, vitamins, and polyphenols. In particular, polyphenols contribute to the health-promoting effects of this food crop, and their levels are influenced by environmental conditions. Production of quinoa is recently being explored in temperate climate areas, including Italy. The aim of this research was to assess the profile of bioactive compounds in seeds of two quinoa varieties, Regalona-Baer and Titicaca, grown in northern Italy, compared to that of seeds of those varieties grown in Chile and Denmark, respectively. High-performance liquid chromatography-diode array detector (HPLC-DAD) analysis of phenolic compounds and *in vitro* antioxidant activity of seed extracts (evaluated by means of three different assays) both in their free and soluble conjugated forms, showed the differences between Regalona grown in Chile and Italy.

During the internship period carried out at the Department of Organic Chemistry at *Universidad Autónoma de Madrid* (UAM), Madrid (ES), it was achieved the isolation of two pentacyclic triterpenoids, from an endemic Peruvian plant, *Jatropha macrantha* Müll. Arg., by applying of bio-guided fractionation technique. The two isolated molecules were subsequently identified as pomolic acid and euscaphic acid and have been proved to be non-cytotoxic on normal cells, while they have been shown to possess inhibitory activity *in vitro* against two transcription factors involved in cancerogenesis. The results of this research project have been published and will be included in the following essay.

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Functional Foods

Abstract

In the last few decades, scientific evidence has pointed out the health-beneficial effects of phenolic compounds present in fruits, vegetables, and grains, and a high intake of these foods has been linked to a lower risk of most common degenerative and chronic diseases that are known to be caused by oxidative stress. In this frame can be inserted research carried out during my PhD thesis, which concerns the phytochemical investigation of phenolic composition in fruits and seeds of food plants, in particular **sweet cherry** (Prunus avium L.), **apple** (Malus domestica L.) fruits and **quinoa** (*Chenopodium quinoa* Willd.) seeds.

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Health and wellness of human beings are intrinsically linked to a proper lifestyle; hence a correct diet rich in nutritious foods is crucial. This concept, however, is not new, in fact the belief that food is a form of medicine for human beings can be traced back to ancient Hindu scriptures and traditional Chinese medicine principles. Hippocrates, who is considered as the Father of Western medicine, 2400 years ago said: "*let food be your medicine and let medicine be your food*", a concept that is still relevant today.

The term "**functional food**" was first introduced in Japan in 1984 to describe a product with health-promoting features. This definition has also been used in Europe and the United States, however causing some confusion among consumers, who may erroneously associate it with the concept of pharmacology rather than nutrition [1]. In 2008, the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE), better clarified the concept from a regulatory point of view, giving the following definition:

"A food can be regarded as "functional" if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern." [2]

and also:

"A functional food can be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means. It can also be a food where the nature of one or more components has been modified, or a food in which the bioavailability of one or more components has been modified, or any combination of these possibilities. A functional food might be functional for all members of a population or for particular groups of the population, which might be defined, for example, by age or by genetic constitution." [2]

Doyon and Labrecque (2008) [3], after screening more than a hundred definitions in the literature, selected the following, that summarized the concept of "functional food" better than others.

"*A functional food is, or appears similar to, a conventional food. It is part of a standard diet and is consumed on a regular basis, in normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions.*" [3]

In other words, functional foods are to be understood as such, and not as pharmaceutical forms (e.g., pills), they are part of a healthy balanced diet and, as a result of the presence of specific compounds, they are beneficial to the health of consumers. These positive effects, in the context of the prevention of chronic diseases or other targets, must have been proven.

Functional foods can be rich in minerals, vitamins, fatty acids, dietary fibers, probiotic and phytochemicals with antioxidant properties, among others. In light of this, plant-derived foods, including fruits and vegetables, represent the simplest form of functional foods [4]. Bioactive compounds that make a food "functional" can be present into the food matrix as a result of two processes:

- → they are naturally present in the food matrix (such as **β-glucans** in oat-derived products, that contribute to lowering levels of serum LDL cholesterol) [5];
- → they were added during the food processing procedures to fortify a certain food (such as **vitamin D** in staple foods or **lactulose**, a synthetic disaccharide, that gives prebiotic qualities to dairy products.) [6,7].

Therefore, some forms of functional foods are included in a normal diet and allow consumers to assimilate bioactive compounds that seems to have a positive impact on health through preventive and protective actions against several types of diseases [4]. Epidemiological studies support a positive correlation between a diet rich in fruits and vegetables and a lower risk of contracting some types of cancer. In particular, many studies support the thesis that the Mediterranean diet (rich in fruits, vegetables and

unsaturated fats, fibers, grains and poor in red and processed meats) can provide a real benefit in terms of chemoprevention, in particular in those forms of breast cancer that develop in premenopausal age [8]. Moreover, the meta-analysis carried out by Guo (2019) on nine independent prospective cohort studies, revealed a strong correlation between consumption of food from plant origin and a reduction (up to 50%) in liver cancer incidence [9]. Also the incidence of colon cancer, which has a high mortality rate, can be mitigated by a plant-based diet, as demonstrated by several epidemiological studies in which the consumption of fruit, vegetables and even legumes, spices and mushrooms has shown protective and preventive activities [10]. The same trend has been highlighted as concerns the risk of developing cardiovascular diseases, which is drastically reduced under high consumptions of fruits and vegetables [11], as well as for other chronic diseases, commonly defined as "**lifestyle diseases**", including obesity, coronary heart disease, type 2 diabetes mellitus, inflammatory disorders [12]. The possible protective activity of an adequate diet has been investigated also for neurodegenerative diseases, but given their complexity, the road to diet-based prevention is yet to be fully explored [13].

In view of this, it is clear that plant-based nutrition (PBN) regimens have gained the attention of the scientific community in the last decades, based on established and emerging scientific evidence as an important means of promoting good health and longevity. PBN requires prevailing consumption of plant-derived whole foods in order to take essential macronutrients (carbohydrates, protein, fats), micronutrients (minerals and vitamins), and phytochemicals (including phenolic compounds, terpenes etc.), able to protect and if possible improve metabolic functions [14]. For this purpose, scientists have begun to modify different kinds of plant-based foods with the aim of improving their nutritional and health-beneficial potentials [15]. Enhancing the nutritional value of plant-based food, either via genetic engineering or breeding, is an interesting and potentially helpful solution to overcoming the global health challenges of nutritional deficiencies and diet-related non-communicable diseases [16].

Dietary phytochemicals

Dietary phytochemicals are a broad group of non-nutritive compounds with different chemical characteristics; with a normal diet in one day, about 500 mg of these are ingested. These substances, even if highly heterogeneous, have some common characteristics: they derive from plant metabolism; they cannot be synthesized by humans; they are not essential; they often have health protection activities when taken at significant levels and have complementary and overlapping mechanisms of action [17]. Plant metabolism is divided into **primary** (or central) and **secondary** (or specialized) metabolism. Primary metabolism provide organic compounds absolutely essential for the plant structure, for its growth and development, and include lipids, nucleic acids, amino acids and carbohydrates [18]. Primary metabolism produces precursors useful for secondary metabolism; this consists of specific and specialized metabolic pathways able to produce organic compounds whose functions support the plant survival through environmental interactions, adaptation and defense responses. The main differences between primary and secondary metabolites reside in their role and in their concentration within plant tissues, the former being produced at much higher concentrations compared to the latter. Moreover, primary metabolites are found in all plant species, while secondary ones are often differentially distributed among distinct taxonomic groups. Indeed, the ability to synthesize secondary compounds has been selected during evolution in different plant lineages when such compounds addressed specific needs. Thus, a much greater diversity is observed in secondary metabolic pathways at the level of species, organs, tissues, cells, and even at different developmental stages [19]. Nevertheless, the boundary between primary and secondary metabolism is getting more and more blurred. For example, a role as transcriptional regulators has been recently demonstrated for flavonoids, a class of phenolic compounds belonging to secondary metabolites, and their capacity of modulating plant growth through their influence on auxin transport has been reported [20]. Because of this, these metabolites are no longer referred to as "*secondary metabolites*", but as "***specialized metabolites***" [21].

These phytochemicals can be divided in four major groups, based on their biosynthetic origin:

- terpenoids,
- phenolics and polyphenolics,
- alkaloids
- sulphur compounds.

They are generally present at lower concentrations compared to primary metabolites, and their localization within plant species can be very different, as well as the function they play. For example, carotenoids are ubiquitous in leaves and stems as they play an essential role in the photosynthetic process, involved in light harvesting and photoprotection [22]; alkaloids are usually concentrated in roots, stems, leaves, fruits, and seeds [23], and due to their bitter taste and toxicity are deterrents against insects and herbivores [24]. Phenolic compounds are ubiquitously, albeit unevenly, distributed in most plant tissues, such as fruits, seeds, leaves, stems, roots and are involved in plant defense against animals, insects, pathogens and in inhibiting the growth of competing plants [25]. Among them, flavonols are preferentially distributed in epidermal cells of leaves and fruits, and they behave as photoprotectors in two different ways: they absorb solar UV radiation and they inactivate UV-generated ROS by acting as antioxidants [26]; moreover, they act as pollinator attractors [27], as phytoalexins [28], as signaling molecules [28], and as regulators of auxin transport [29].

From a structural point of view, phenolic compounds have at least one aromatic ring with one or more hydroxyl groups attached. More than 8000 phenolic structures have been reported so far, and they are widely distributed throughout the plant kingdom, usually conjugated to sugars and organic acids. Phenolic compounds are produced in plants through a complex sequence of biosynthetic steps originating from phenylalanine and/or tyrosine, which in turn are synthetized through the shikimate pathway [30]. The first step of the phenolic compound biosynthetic pathway begins with the deamination of phenylalanine to *trans*-cinnamic acid (fig. 1), catalyzed by the enzyme PAL (Phenylalanine Ammonia-Lyase).

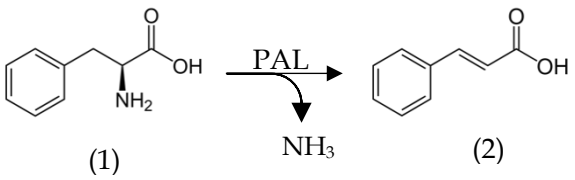

Figure 1: Deamination of phenylalanine (1) by PAL (phenylalanine ammonia-lyase) enzyme to trans-cinnamic acid (2).

This metabolic step is crucial because *trans*-cinnamic acid will be the precursor of many other phenolic compounds [25]. According to their biosynthetic origins and molecular structure, phenolic compounds can be categorized into two sub-groups: ***flavonoids*** and ***non-flavonoids***.

Flavonoids

Flavonoids are structurally composed of fifteen carbon atoms, with two aromatic rings connected by an heterocyclic (usually a 2*H*- or 4*H*-pyran) bridge (fig. 2). The basic flavonoid skeleton can have several substituents; hydroxyl groups are usually present at the C3, C5, C7 and C4' positions.

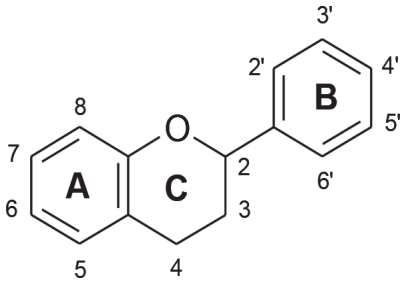

Figure 2: The basic flavonoid structure

Major flavonoids can be divided in six subclasses, according to differences in the pyran ring: flavonols, flavones, isoflavones, flavan-3-ols, flavanones and anthocyanidins (fig.

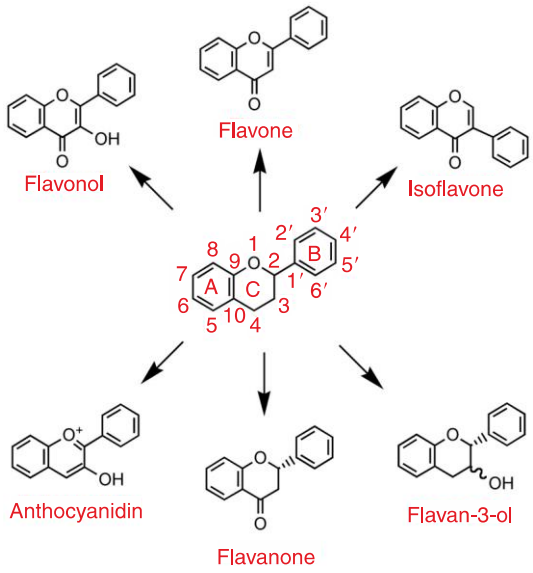

Figure 3: The six flavonoid subclasses, which all share a flavan nucleus.

3). In each of them, individual compounds differ in their pattern of hydroxylation and methylation of the A and B rings. Minor flavonoids comprise dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones [31]. Most flavonoids occur in plant tissues as glycosides, bound to sugar moieties, and only a few of them exist as aglycones in nature. The sugar moiety gives to the molecule more hydrophilicity. *O*-glycosylation and C-glycosylation represent the main conjugation forms; in the latter, the glycoside moiety is linked to the aglycone structure with a C–C bond, and this conjugation mostly occurs in C6 and C8 [32]. *O*-glycosylation is much more frequent, with the sugar molecule mostly attached to free hydroxyl groups in the A and C rings via a β-glycosidic bond [32].

As a general rule, D-configured sugars, i.e. glucose, galactose, xylose, and glucuronic acid form β-bonds, while α-bonds are made by L-sugars, such as arabinose and rhamnose

[33]. In particular, flavonols occur as 3-*O*-glycosides, while other subclasses also include 7-*O*-glycosides [32]. They are distributed mainly in leaves and in fruits peel and are involved in different processes such as UV protection, pigmentation, stimulation of the nitrogen-fixing nodules and resistance to diseases [26]. From a biosynthetic point of view, the characteristic flavonoid nucleus is obtained by the two enzymes chalcone synthase (CHS) and chalcone isomerase (CHI), which catalyze the condensation of three molecules of malonyl-CoA with 4-coumaroyl-CoA into the flavanone naringenin [25,34] (fig. 4).

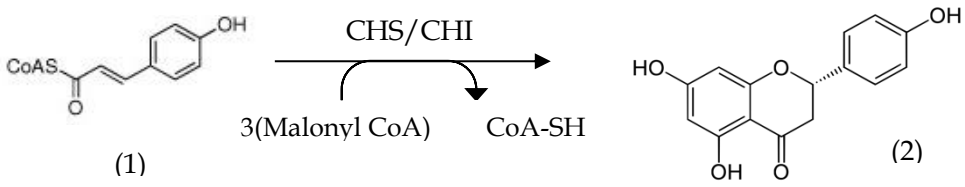

Figure 4: The formation of flavanone naringenin (2) from 4-coumaroyl CoA (1), by enzymes chalcone synthase, CHS and chalcone isomerase, CHI.

From this point on, naringenin is a key intermediate because the remaining biosynthetic flavonoid pathway branches off into each of the six major subclasses: isoflavones, flavanones, flavones, flavonols, flavan-3-ols and anthocyanins [31] as summarized in fig. 5.

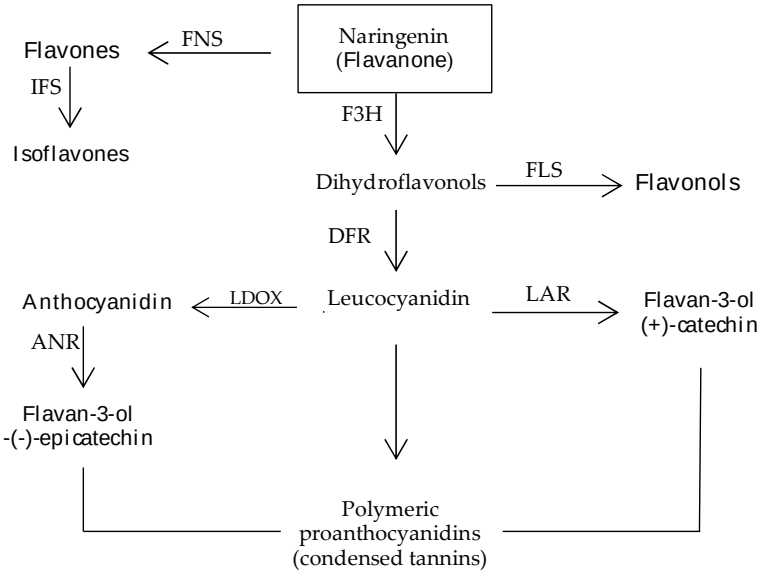

Figure 5: Flavonoids biosynthetic pathways. Enzymes abbreviations: FNS, flavone synthase; IFS, isoflavone synthase; F3H, flavonone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LDOX, leucocyanidin deoxygenase, LAR, leucoanthocyanidin 4-reductase; ANR, anthocyanin reductase.

Flavonols

Among flavonoids, flavonols are the most represented subclass. Structurally, flavonols are characterized by the typical flavan nucleus (C6-C3-C6) as well as a double bond between C2-C3, a hydroxyl in C3 and a carbonyl in C4. They exist as aglycones, but they can also be found as glycosides, sulfonated, and methylated derivatives.

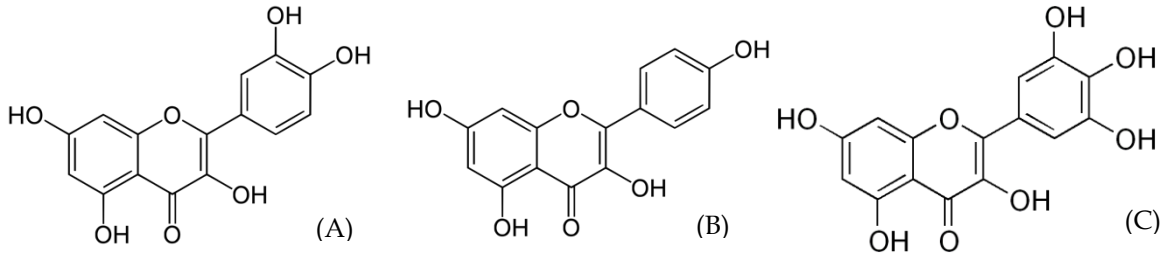

Figure 6: The flavonol aglycones quercetin (A), kaempferol (B) and myricetin (C).

The most important aglycones are quercetin, kaempferol, myricetin, (fig. 6) although in plants they mostly occur as glycosides, the most suitable form for storage in aqueous cell vacuoles [35,36].

Glycosylated flavonols usually have glucose, rhamnose, galactose, arabinose, xylose, and glucuronic acid as sugar moiety [37]; however, acylation also takes place with different acids, such as acetic, glutaric, glucuronic, oxalic and caffeic ones [38]. Conjugation occurs most frequently at the C3 position of the C-ring, but can also be found at the 5 and 7 positions of the A-ring as well as 4′, 3′ and 5′ positions of the B-ring. Fruits and vegetables are important dietary sources of *O*-glycosylated flavonols: berries, onions, Cruciferae vegetables, buckwheat, tea and red wine are rich in flavonols, in glycosylated form and as aglycones [33]. Since their biosynthesis is stimulated by light irradiation, these metabolites are mainly present in leaves, flowers, and outer parts of plants (such as the peel of the fruits) and decrease in concentration toward the innermost tissues [39]. They are also subjected to significant variations due to seasonal influences, as has been demonstrated in some types of leafy vegetables and fruits. In summer, flavonol concentrations can be three to five times higher than in other seasons [39]. Quantification of these compounds can be challenging, because of the large number of glycosylated derivatives, therefore an acid hydrolysis is often carried out to break the glycosidic bond and release more easily identifiable aglycones [40]. From a nutritional point of view, it is

important to note that only a small fraction of flavonol aglycones are absorbed as they are, because they are present mainly as glycosidic derivatives, which differ in sugar moieties.

Several quercetin derivatives occur in nature, such as rutin, quercitrin, and hyperoside, among others, deriving from its conjugation with rutinose, rhamnose, and galactose, respectively [41]. Quercetin is certainly one of the most represented and present flavonols in food, its intake in the Caucasian diet can reach 30 mg per day, thanks to the consumption of fruits (mainly berries and grapes) and vegetables, as well as tea. Besides quercetin, kaempferol is also an important dietary flavonol, quite ubiquitous in fruits, vegetables, and tea; It also occurs mainly bonded to sugars such as glucose and rhamnose (two units) [42].

Flavones

Structurally, what distinguishes flavonols from flavones, is the lack of an hydroxyl group on C3. Hydroxylation, methylation, *O*- and *C*-alkylation, and glycosylation are possible substitutions. Most flavones occur as 7-*O*-glycosides, but also as acetyl or malonyl moieties. *C*-glycosides flavones are generally 6-C-and 8-C. The sugar moieties include glucose, rhamnose, galactose, xylose and arabinose [43]. While *O*-glycoside flavones can be hydrolyzed with enzymes or acids prior to analysis, *C*-glycoside flavone are resistant to most hydrolysis methods and must be evaluated in their native form [44]. Flavones are not very abundant in nature, the only exceptions being parsley, celery and chamomile, which are especially rich in apigenin and luteolin. In particular, several derivatives of apigenin, such as acetyl, malonyl, and caffeoyl have been detected in chamomile [44]. Polymethoxylated flavones (nobiletin and tangeretin) have been found in the fruit peel of different *Citrus* species [31] (fig. 7).

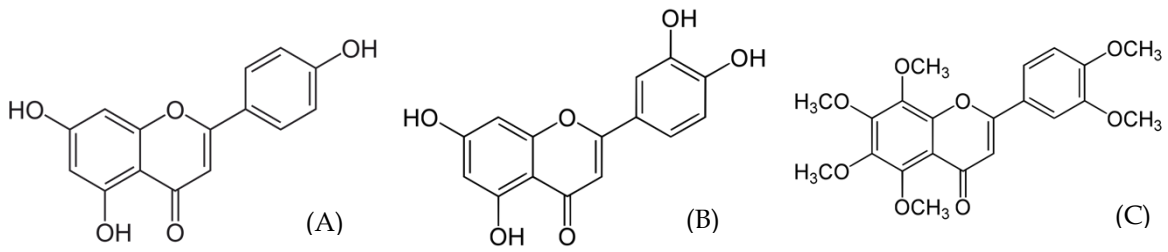

Figure 7: The flavone aglycones apigenin (A), luteolin (B) and the polymethoxylated nobiletin (C).

Flavan-3-ols

Structurally, flavan-3-ols are the most complex subclass of flavonoids. They lack the double bond in C2-C3 and the chetonic group in C4, while a hydroxylic substituent is present at C3 [25]. They include either monomeric structures, such as catechin and epicatechin, or oligomeric and polymeric proanthocyanidins, also called condensed tannins. The structure is not planar because of the saturated C2-C3 element in the heterocyclic ring C. The two chiral centers at C2 and C3 of the flavan-3-ol molecule produce four isomers for each level of B-ring hydroxylation, two of which, (+)-catechin and (−)-epicatechin (fig. 8), are widespread in nature. Contrarily to the other classes of flavonoids, they are rarely found in glycosidic form [31].

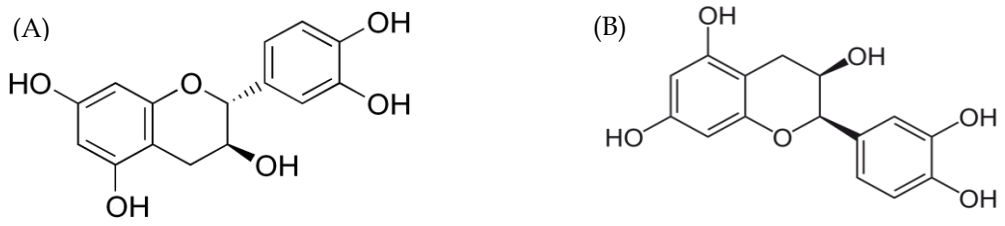

Figure 8: The flavan-3-ol (+)-catechin (A) and (−)-epicatechin (B).

Fruits (mostly berries, peaches and apples) and vegetables are sources of monomeric flavan-3-ols, while proanthocyanidins are found in tea, wine, cereals and nuts [25], and their presence in foods is responsible for organoleptic characteristics (color, astringency, bitterness, sweetness, aroma) [45].

Anthocyanidins

This subclass of flavonoids lacks the carbonyl group in C4 and possesses one hydroxyl in C3 and a double bond in the ring C, resulting in the only ionic flavonoids [25]. Anthocyanidins as well as their derivatives are found in many plant tissues (leaves, stem, seeds and roots), but mainly in flowers and fruits. Thanks to their backbone structure, the flavylium cation, they are water-soluble pigments responsible for the violet, red and blue color of these organs and their roles in plant tissue are photoprotective, antioxidant and attractive towards pollinators.

The aglycones are called anthocyanidins, and are divided into 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and O-methylated anthocyanidins; both the glycosylated and the acylated derivatives are called anthocyanins. Glycosylation improves water stability and solubility of anthocyanidins, and the most common linked sugar are glucose, galactose, rhamnose, arabinose, rutinose, sambubiose, and sophorose [46]. In addition, sugar residues can be acylated with hydroxycinnamic acids (*p*-coumaric, caffeic, and ferulic acids), hydroxybenzoic acids (*p*-hydroxybenzoic acid) or other aliphatic acids (malonic, succinic, malic, and acetic acids) [47]. The highest concentration of anthocyanins in the human diet comes from berries of the *Vaccinium*, *Ribes*, *Prunus* and *Sambucus* genera [48]. The most representative types of anthocyanidins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin [49] (fig. 9), although more than 25 have been identified.

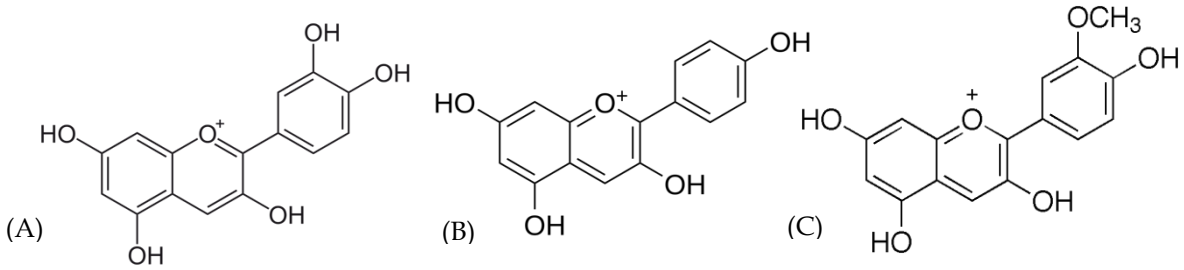

Figure 9: Structures of anthocyanidins: cyanidin (A), pelargonidin (B), peonidin (C).

As concerns anthocyanins, more than 600 compounds have been found, such as cyanidin derivatives (cyanidin-3-*O*-rutinoside or keracyanine; cyanidin-3-*O*-glucoside or kuromanin) and peonidin derivatives (peonidin-3-*O*-rutinoside and peonidin-3-*O*-glucoside) [50].

Flavanones

Flavanones are the key intermediate in the biosynthetic pathway of phenylpropanoids. They lack the double bond between C2 and C3, which is present in flavones and flavonols, and show a carbonylic group in C4 [51]. The flavanone structure is highly reactive and has been reported to undergo hydroxylation, glycosylation and *O*-methylation. The most representative aglycones are hesperetin, naringenin (fig. 10), eriodictyol, isosakuranetin and taxifolin, even though flavanones are mostly found as their glycosylated derivatives.

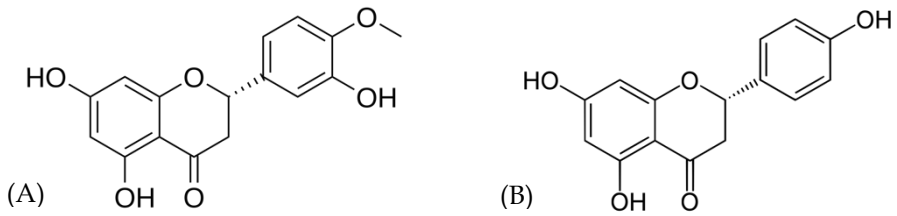

Figure 10: The flavanones aglycones hesperetin (A) and naringenin (B).

The latter group includes several compounds with a sugar moiety bound to the aglycone hydroxyl groups, through an *O*-glycosidic linkage in C7 position in the A ring. The bound saccharide is generally either a rutinose or a neohesperidose moiety [52].

Isoflavones

Isoflavones are the only subclass of flavonoids characterized by the position of the B ring in C3 rather than in C2. These compounds are mainly, but not exclusively, found in plants belonging to the Fabaceae family. Soybeans (*Glycine max* L. Merr) are the richest source of isoflavones, including daidzein, genistein and glycitein, while red clover (*Trifolium pratense* L.) is rich in formononetin and biochanin A. Isoflavones are referred to as phytoestrogens, because they are structurally similar to the steroid hormone 17β-estradiol, (fig. 11) thus mimicking its activity.

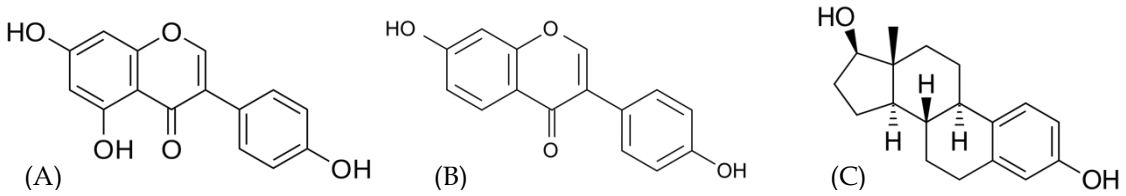

Figure 11: The isoflavones genistein (A) and daidzein (B); structure of 17β-estradiol (C).

Isoflavones are known to be chemoprotective [53] and show *in vitro* antiproliferative activity against several cancer cell lines [54,55]. The dietary intake of isoflavones in food, as well as in the form of pure compounds in pharmaceutical form, has shown positive effects in the treatment of many diseases: cardiovascular [56], osteoporosis [57], menopausal syndromes [58].

Minor flavonoids: dihydrochalcones

Some minor flavonoids include dihydroflavones, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones [31]. Despite their lower abundance compared to the major subclasses, some of them can play important roles not only for plants, but also for animals and humans [59]. Dihydrochalcones are characterized by an open C-ring structure; they can be found either in the free form, or as C-glycosylated, C-benzylated or prenylated derivatives. In the plant kingdom they are mainly found in apple trees (*Malus x domestica* Borkh., *Malus* sp., Rosaceae) [60]. Phloretin, as well as its glycoside conjugates (phloretin-2′-*O*-glucoside or **phloridzin** and phloretin 2′-*O*-(2″-*O*-xylosyl)-glucoside) (fig. 12) have been the subject of numerous studies. Indeed, they are among the main phytochemical markers of the apple, and good health-promoting potentials have been demonstrated, such as *in vivo* and *in vitro* hypoglycemic effects [61].

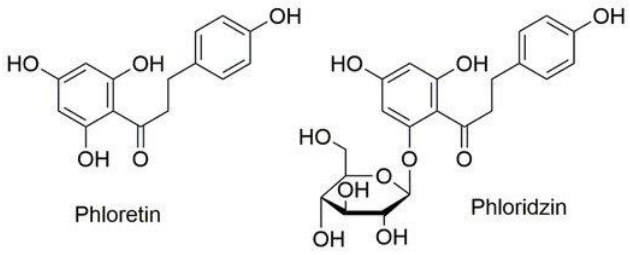

Figure 12: Structures of phloretin and phlorizin (phloretin 2'-O-glucose)

Non-flavonoids

The major non-flavonoids relevant to nutrition are: C6–C1 phenolic acids, in particular gallic acid, which is the precursor of hydrolysable tannins, C6–C3 hydroxycinnamates and their conjugated derivatives, and polyphenolic C6–C2–C6 stilbenes [25,31].

Hydroxybenzoic acids possess a C6–C1 structure, which derives from benzoic acid. Variations in structure are mainly hydroxylations and methoxylations of the aromatic ring. The most common compounds belonging to this group are gallic, *p*-hydroxybenzoic, vanillic, syringic, and protocatechuic acids (fig. 13), they are rarely found in free form, since they are usually conjugated with organic acids (quinic, maleic,

tartaric acids) or with structural components of the plant cell wall (cellulose, proteins and lignin), from which they can be released by alkaline hydrolysis [62].

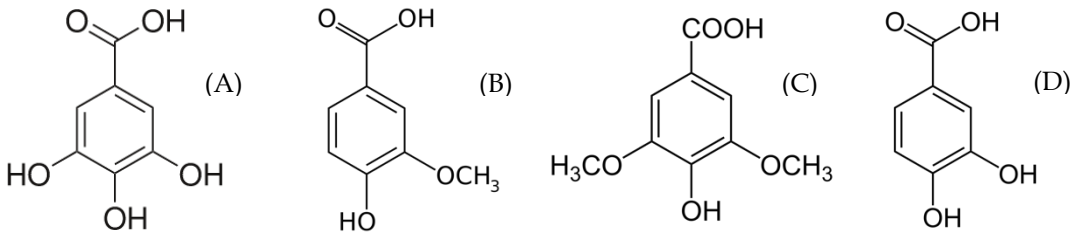

Figure 13: Hydroxybenzoic acid structures, gallic acid (A) vanillic acid (B), syringic acid (C) and protocatechuic acid (D).

Gallic acid is found both in free form and as a base unit of hydrolysable tannins called gallotannins, whereas ellagic acid and hexahydroxydiphenoyl moieties are both subunits of the ellagitannins. The term "*hydrolyzable tannins*" therefore includes both gallotannins and ellagitannins, which easily release gallic or ellagic acid, respectively, after acidic hydrolysis.

Both condensed and hydrolysable tannins, are responsible for protein precipitation, especially collagen. They tend to bind with saliva, giving a taste defined astringent, typical of certain foods such as tea and wine [31]. Gallic acid is the precursor of the hydroxybenzoate class and originates directly from the shikimic acid route; subsequently, it undergoes further galloylation reactions with consequent synthesis of gallotannins and ellagitannins, or hydrolysable tannins [31].

Hydroxycinnamic acids are characterized by a lateral chain with three carbon atoms (C6-C3); the most common ones are cinnamic, *p*-coumaric, caffeic, ferulic, sinapic acids (fig. 14).

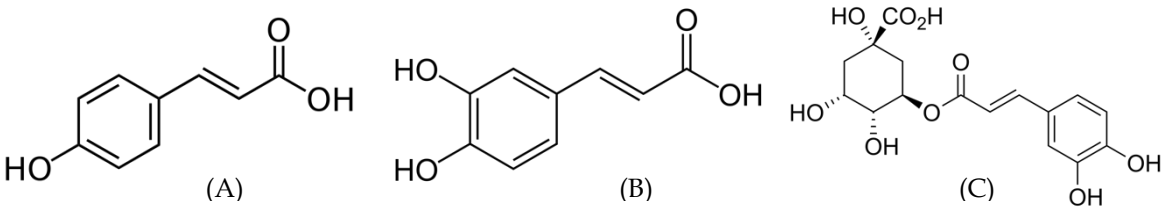

Figure 14: Hydroxycinnamic acids, p-coumaric acid (A), caffeic acid (B) and chlorogenic acid (C).

The hydroxycinnamates occur mainly as conjugates, for example, with tartaric or quinic acid. Quinic acid conjugates of caffeic acid, such as 3-, 4- and 5-*O*-caffeoylquinic acid, are widespread in fruits and vegetables, in particular sweet cherries are rich in the first isomer also known as **neochlorogenic acid** [63]. 5-*O*-caffeoylquinic acid is probably the most abundant soluble hydroxycinnamic acid derivative, it is also found in tobacco and coffee, and is very often referred as chlorogenic acid, although this term includes a group of related compounds [31,64]. Hydroxycinnamic acids derive from the synthetic intermediates that run from *trans*-cinnamic acid (produced by phenylalanine) to naringenin. As shown in figure 15, starting from cinnamic acid with a series of enzymatic reactions, sinapic acid is obtained; caffeic acid comes from *p*-cumaric acid and after a 3-*O*-methylation it becomes ferulic acid. And finally, chlorogenic acids, which includes isomers 4-*O*-caffeoylquinic acid (cryptochlorogenic acid or 4-CQA) and 5-*O*-caffeoylquinic acid (neochlorogenic acid or 5-CQA) derive from the intermediate *p*-coumaroyl CoA through multiple enzymatic reactions.

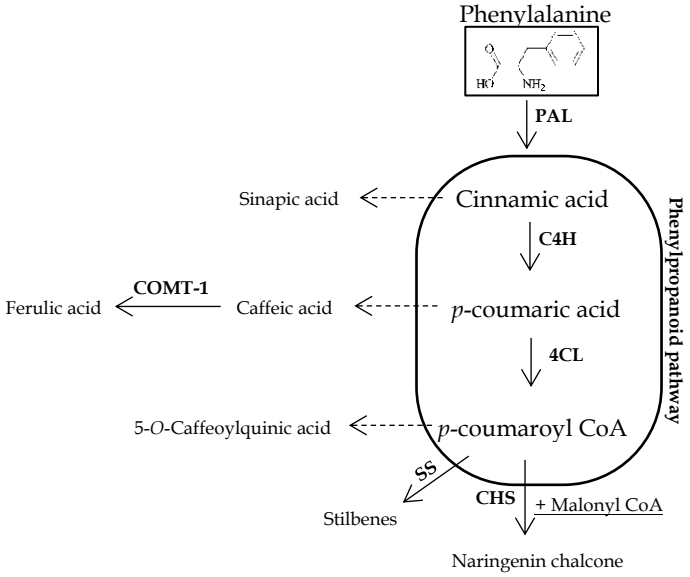

Figure 15: Phenylpropanoid pathway, from phenylalanine to hydroxycinnamic acids. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase, 4CL p-coumarate:CoA ligase, COMT-1, COMT-1, caffeic/5-hydroxyferulic acid O-methyltransferase, SS, stilbene synthase.

Stilbenes

Stilbenes are characterized by a C6–C2–C6 structure and tend to accumulate in plants both in free and glycosylated forms, as well as in methoxylated or oligomer forms. Only a small range of plant species can synthesize these compounds, and always as a response to various biotic (pathogen or herbivore attack) and abiotic stresses (environmental stresses such as cold, drought, salinity, ultraviolet light, and ozone exposures). Resveratrol (fig. 16) is the most common and investigated stilbene; the interest in monitoring its levels in red wine is mainly due to the "*French paradox*" (the observation that a moderate consumption of red wine may reduce the risk of coronary heart diseases) [65]. High concentration of resveratrol can also be found in Japanese knotweed, *Poligonium cuspidatum* Siebold & Zucc. (an ancient herb used in traditional Asian medicine) as well as peanuts and berries [66]. These compounds are produced through the phenylpropanoid pathway by the action of the stilbene synthase (SS) enzyme that catalyzes the formation of the stilbene structure from *p*-coumaroyl-CoA, which is also the precursor of flavonoid pathways [67] (fig. 15).

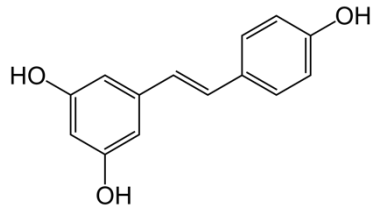

Figure 16: Structure of resveratrol.

C*hemical forms of polyphenols in plant tissues*

As described above, phenolic compounds occur in plant tissues in different chemical forms, and they can be classified as free, soluble-conjugates and insoluble-bound, respectively [68,69]. The free forms of phenolic compounds are present in small amounts

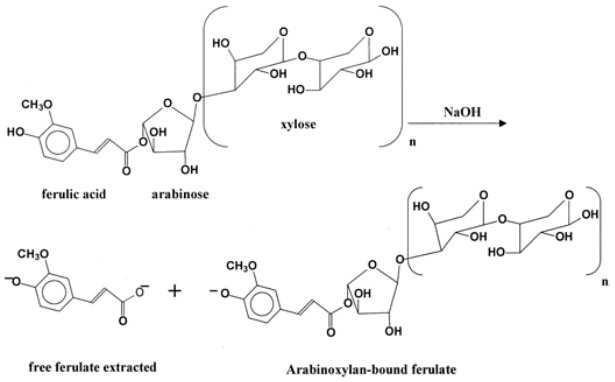

Figure 17: Example of alkaline hydrolysis of bound ferulic acid that releases the free phenolic acid, that subsequently will be protonated with acid, to facilitate extraction with polar solvents.

in plants. They occur in much larger ratios as esters, glycosides, and bound complexes. Soluble-conjugated phenolic compounds form covalent bonds with low molecular weight cellular components, while the insoluble forms include those phenolics covalently bound to cell wall structural components such as cellulose, hemicelluloses (e.g., arabinoxylans), lignin, pectins and rod-shaped structural proteins. Phenolic acids, such as hydroxycinnamic and hydroxybenzoic acids, form both ether linkages with lignin through their hydroxyl groups, and ester linkages with structural carbohydrates and proteins through their carboxylic group. As already mentioned, flavonoids are mainly present as glycosides, linked to a single or multiple sugar moieties. While free phenolic acids are easily extractable from the plant matrix through several methods using mixtures of solvents, a preliminary step is necessary to analyze the conjugated forms [70]. An acidic or alkaline hydrolysis is a widely used procedure in the identification of many phenolic compounds. For bound phenolic acids, treatment with a strong base at room temperature for a prolonged period is indicated, to break down the ester bond that links the phenolic acid to the other components (components of the cell wall, polysaccharides) [68] (fig. 17).

Subsequently, the obtained phenolics are extracted with polar solvents (ethyl acetate, diethyleter) with a biphasic separation procedure [70]. Acid hydrolysis is used instead to quantify aglycone flavonoids, as the acid (mainly hydrochloric acid) breaks the glycosidic bond, releasing the molecule from the sugar moiety (fig 18).

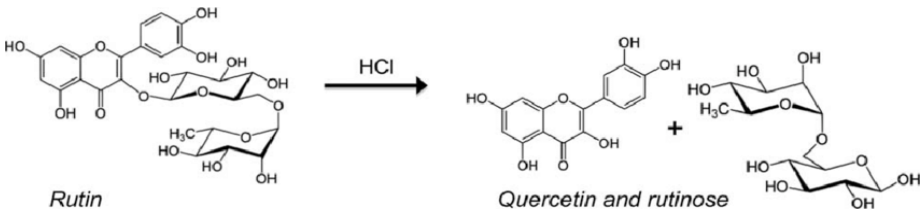

Figure 18: Quercetin-3-O-rutinoside (or rutin) treated with concentrated hydrochloric acid, release aglycone quercetin and the sugar moiety, by breaking glycosidic bond.

Since this type of treatment is very aggressive and could damage phenolic acids, it is often applied as a step after basic hydrolysis, so that the phenolic acid portion has already been extracted [68]. Depending on the compound of interest, it is necessary to choose the

most suitable extraction process [71]. The presence of the three forms of phenolic compounds (free, soluble-conjugated and insoluble-bound) in plants is quite variable, it is possible indeed to find them in all three forms, but in very different quantities. All the three types of phenolic compounds have been identified in fruits, even if the insoluble bound fraction could only be found in a small ratio [72]. On the contrary, in cereal grains, the bound phenolic fraction constitutes the largest portion (85% in maize, 75% in oats, 62% in rice) and contributes for the most part to the antioxidant capacity of the food. Free phenols are also found, even if in lower quantity.

Polyphenols and antioxidant capacity

Molecular oxygen (O_2) is intrinsically linked to the survival of all aerobic organisms. Thanks to its high reactivity, it can generate energy (in the form of adenosine-5-triphosphate, ATP) through oxidative phosphorylation of organic compounds. ATP synthesis occurs in mitochondria and stems from the gradual release of electrons to oxygen, each time generating reactive oxygen species (ROS) also known as "free radicals", which if not properly intercepted and neutralized, can be released into the mitochondrion with consequent damage. They are defined as "unstable molecules that contain oxygen and that easily react with other molecules in a cell. A buildup of reactive oxygen species in cells may cause damage to DNA, RNA, and proteins, and may cause cell death" [73].

The most representative among the various types of ROS, are certainly: the superoxide anion $O_2^{\bullet -}$, hydrogen peroxide H_2O_2 and the hydroxyl radical $\bullet OH$, although there are many others. They are mainly produced in mitochondria under physiological and pathological conditions, stimulated from exogenous (ionizing radiations, ethanol, xenobiotics, smoke and environmental pollution) and endogenous factors (cellular respiration, lipid metabolism, enzymatic activity) [74]. The imbalance between the production and accumulation of radicals in cells and tissues and their inactivation is a condition known as oxidative stress. The most serious damage caused by free radicals, and especially the hydroxyl radical, is that concerning DNA. They can damage nucleobases and, if not properly identified and repaired, the damage will be transmitted to future generations. Proteins and lipids are also preferred targets for free radicals.

The oxidation of amino acids causes a structural alteration and subsequent loss of biological function. Membrane phospholipids are often attacked by ROS, causing a loss of fluidity and permeability of cell barriers. The organism must therefore face the danger represented by free radicals, using the proper mechanisms of endogenous defense, either through the primary (or enzymatic) antioxidants or through exogenous molecules able to restore the balance within the cells [75]. An antioxidant is indeed defined as any molecule that is able, at low concentrations, to significantly delay or inhibit the oxidation of a substrate. The antioxidant machinery in cells comprises both enzymatic and non-enzymatic processes.

The primary antioxidant defenses of cells reside in enzymatic activity capable of neutralizing superoxides and hydroperoxides, as follows:

- Initially, superoxide dismutase (SOD) a family of metalloenzymes, catalyze the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen:

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

- The resulting hydrogen peroxide is neutralized by the action of the enzyme catalase, which converts hydrogen peroxide into water and molecular oxygen:

$$H_2O_2 \rightarrow 2H_2O + O_2$$

- Cytosolic glutathione peroxidase (GPX1 in particular) is also able to neutralize hydrogen peroxide. However, this enzyme needs secondary enzymes and cofactors to function properly:

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$ [76]

The group of non-enzymatic antioxidants include compounds that are usually absorbed with the diet: hydrophilic vitamins (ascorbic acid), lipophilic vitamins (tocopherols, retinols, vitamin K), mineral salts (selenium and zinc ions), enzymatic cofactors (Q_{10}), peptides and polyphenols (flavonoids and phenolic acids) [77]. It is now clear that phenolic compounds are endowed with a variable and strong capacity to act as antioxidant agents inhibiting the auto-oxidation reactions and behaving as free radical

scavengers. Even though the relationship between the chemical structure of these compounds and their antioxidant activity has not yet been fully clarified, some conclusions have been drawn. Some flavonoids (such as tea catechins) have shown a chelating ability against metals involved in oxygen metabolism, such as iron and copper, which reduce hydrogen peroxide, generating the hydroxyl radical and then oxidizing low-density lipoproteins; the functional groups related to chelating activity, are the catechol function and the hydroxyl groups in C3 and C3' [78].

Catechol indeed is able to produce very stable radical species, mainly due to the delocalization of the odd electron on the aromatic ring by resonance. From a structural point of view, it has also been shown that the antioxidant activity of flavonoids is deeply influenced by their functional groups [79] (fig. 19).

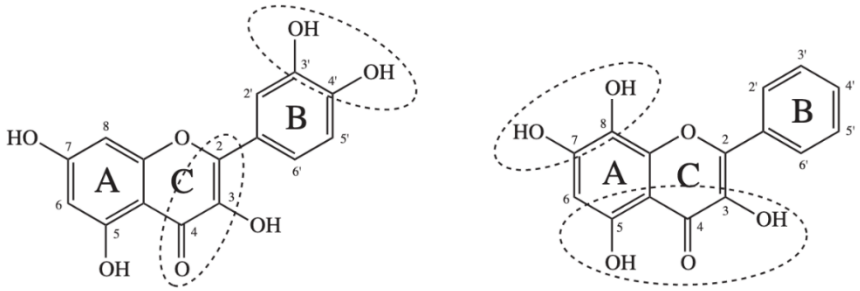

Figure 19: Graphic examples of structure-activity relationships of flavonoids antioxidant power.

- There will be higher antioxidant power, when the ring B consists of a catechol, as it turns out to be a better electron-donor group than a simple phenol, as already explained [80].
- Moreover, to achieve best activity, a double bond in position C2-C3 is necessary and it should be conjugated with a carbonyl group in C4, which is responsible for electron delocalization [79,80].
- The presence of both -OH groups in C3 and C5 dramatically increases radical scavenging ability [81].
- In the absence of a catecholic structure, hydroxyl substituents in the A ring tend to compensate and become determinant for antiradical activity [82,83].

In light of these considerations, it is clear that flavonols and flavones, which possess a catechol function, are the polyphenols most active as antioxidants; in particular flavonols, due to the presence of an -OH group in C3. Quercetin for example, have been reported to be more antioxidant than luteolin [81]. Rutin instead, having the -OH group engaged in a glycosidic bond, is reported to have a reduced radical-scavenging activity compared to its aglycone. The presence of an additional -OH group in the B ring (pyrogallol group), as in myricetin, increases the radical-scavenging activity compared to other flavonoids; on the contrary, the presence of only one -OH group in the same ring, as in kaempferol, decreases the antioxidant activity [83]. With regard to the structure-activity relationships of phenolic acids, it is important to remember that they belong to two chemically different subgroups. The antioxidant capacity of hydroxycinnamic acids is related to their ability to neutralize radicals, bind proteins, and chelate metals, together with their greater reducing power.

The presence of a further -OH in *ortho-/para-* position increases antioxidant capacity, by stabilizing a resonance system and forming related quinones; moreover, the number and position of hydroxyl groups play the most important role in determining the highest activity; from this will result that caffeic acid and related esters will have higher antioxidant capacity than ferulic acid, and in addition caffeic acid dimers (such as rosmarinic acid) will have an increased activity compared to the monomer [84,85]. Chen (2020) proposed an analysis of the structure-activity relationships (SAR) of six phenolic hydroxybenzoic and six hydroxycinnamic acids, using several *in vitro* assays and computational methods. First of all, being equal the number and type of substituents on the benzoic ring, hydroxycinnamic acids (-CH=CHCOOH) are more powerful than hydroxybenzoic acids (-COOH), since the latter has a lower electron-donability. In addition, the larger the number of methoxyl substituents, the greater the antioxidant activity of phenolic acids, as well as flavonoids and stilbenes [86].

Nowadays, there is a rising interest in the detection and quantification of antioxidant capacity of plant-derived food, in the search of natural and effective antioxidants to combat pathological complications related to free radical formation. Antioxidant assays therefore play a crucial role in evaluating the antioxidant capacity of natural products. To date, antioxidant capacity is measured using test panels where each test has its own advantages and limitations. Antioxidant assays are based on the concept of Total

Antioxidant Capacity (TAC), calculated as the amount of free radicals extinguished by the test solution used to assess the antioxidant potential of the sample. Depending on the mechanism of the chemical reactions involved, TAC assays can be more specifically classified as: **single electron transfer** (SET); **hydrogen atom transfer** (HAT); **chelation of transition metals**.

SET-based methods detect the ability of an antioxidant compound to reduce a substrate by electron transfer; this is done visually with a color change within the sample as soon as oxidation is inhibited or decreased [87]. HAT-based methods, instead, measure the ability of an antioxidant compound to quench free radicals with a donation of hydrogen atoms; these reactions are quite rapid and simple, however they are not free from errors by contamination of reducing compounds (metals for example) [88]. In addition, an antioxidant can also act through the ability to chelate transition metals, namely Zn^{2+}, Fe^{2+} and Cu^{2+}. This mechanism is of particular relevance, because multiple studies have shown that transition metals are involved in the pathogenesis of several diseases, such as neurodegenerative and cardiovascular illness [89].

Given the complexity of plant/biological matrices, antioxidant activity cannot be simply measured with a single assay, the choice of the right tests must be pondered in order to obtain results that are as much plausible and complete as possible. The available assays are many, and are divided into three macro categories: spectrophotometric, electrochemical and chromatographic. Those belonging to the first group are certainly the most applied, and among them, are distinguished by the principle of the method (choice of the radical, for example) and by the choice of the determination method of the final product (colorimetry, fluorescence, chemiluminescence) [89].

- **DPPH** assay exploits the use of the 2,2-diphenyl-1-picrylhydrazyl radical, which is very stable, thanks to its remarkable ability of electronic delocalization, and is characterized by the purple coloration of the solution. In the assay, the latter is neutralized by an antioxidant agent accepting a hydrogen or an electron, leading to the formation of reduced DPPH, which is colorless. This reaction is generally evaluated in organic media such as ethanol or methanol. It is a very easy and cheap method; however, it is not free from false results due to the interaction with other molecules and is sensitive to light [87,90]. It is important to underline that there is

no physiological or structural similarity between the DPPH radical and the radicals found in the human body [89].

- **ABTS** assay, also called as TEAC (Trolox Equivalent Antioxidant Capacity) assay, uses 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical, which is generated by a chemical reaction, characterized by an intense blue color [90]. When the ABTS radical accepts one electron from the antioxidant agent, the solid blue color fades into a much paler hue, which means the regeneration of stable form of ABTS. The advantages of this assay are ease of execution, high reproducibility, stability of the radical and good correlation with phenolic compounds; it is also conducted in an aqueous environment. Even in this case, however, the radical structure is not comparable to physiological ones and there is the additional step of its preparation [87,89].

In both assays, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an hydrosoluble analog of vitamin E, is often used as a reference antioxidant, evaluating the percentage of discoloration obtained with the sample in relation to that obtained with Trolox.

Although these two assays are usually classified as SET-based, these two radical indicators can also be neutralized by radical extinction by H-atom transfer (HAT-based). The following assay, on the contrary, is entirely HAT-based [88].

- **ORAC** (oxygen radical absorbance capacity) assay is performed using a radical initiator, 2,2′-azobis(2-amidinepropane) dihydrochloride (AAPH), to produce peroxyl radicals that react with fluorescein (3′,6′-dihydroxy-3H-spiro[2-benzofuran-1,9′-xanthen]-3-one), thus decreasing its fluorescence. The presence of an antioxidant delays the fluorescence decrease by inhibiting radical-induced oxidation through H atom transfer. Antioxidant capacity evaluation is carried out by the integrated areas under the time/fluorescence decay curves. The results are compared with those of a reference antioxidant, usually Trolox. This assay is designed to simulate cellular oxidative processes, and thus is usually performed at 37 °C [91]. It offers many advantages over the previous ones: the radical used is a similar to what is found *in vivo*; the temperature is physiological (37°C); it can be

easily adapted to evaluate hydrophilic and lipophilic molecules; moreover, it can be easily automated. The complexity in the preparation of the reaction is a major limit, variations in temperature and concentration of the probe can adversely affect the result, as well as the treatment of the radical initiator [92].

Polyphenol bioavailability

The concept of "**bioavailability**" of food or nutrients is expressed as the quantity and speed with which they (or their parts) are digested, absorbed and metabolized through several pathways [93]. This process involves several steps, including the release of the active ingredient from the matrix to which it belongs, absorption, distribution, metabolism and finally elimination. Bioaccessibility, is tightly linked to bioavailability, and indicates the fraction of a nutrient that is released from the food matrix in the gastrointestinal lumen and thus made accessible for intestinal absorption: food matrix effect, transporters, molecular structures and enzymatic activity are factors that can affect it. Bioavailability therefore becomes a key concept when it comes to dietary intake of natural substances to ensure their impact on the promotion of human health. In the case of polyphenol compounds, their extreme structural multiplicity still makes difficult to individuate common mechanisms that could ensure their bioavailability [94].

Polyphenols are usually characterized by a low oral bioavailability, mostly due to mass metabolism occurring by both phase I and phase II reactions in enterocytes and liver but also in the gut microbiota [94,95]. They can be absorbed into the gastrointestinal tract after being released from the food matrix, hence becoming bioaccessible. Their uptake may be influenced by solubility (lipophilic and hydrophilic features result in different absorption mechanisms), interaction with some other food constituents, biochemical transformation, different cellular transporters, metabolism, and interaction with gut microbiota [95]. As mentioned above, polyphenols in fruits and vegetables are rarely found in free form, they are more commonly found as soluble conjugates (esterified or glycosylated).

However, these more complex molecules cannot be absorbed as such by the organism, as they require prior hydrolysis. After ingestion, a portion of phenolic compound glycosides

may already be hydrolyzed by β-glycosidase salivary enzymes, however its effectiveness is dependent on the types of sugars present in the molecule: glucose conjugates are indeed rapidly hydrolyzed contrary to rhamnose ones [96].

The main site dedicated to transformation and absorption of phenolics is the small intestine [97]. After being absorbed, phenolic compounds follow the usual metabolic pathway of exogenous substances and, like drugs and other xenobiotics, are subjected to phase II metabolism, then the metabolites reach the liver through the portal vein, where they can undergo further changes (phase I and II metabolism), before entering the bloodstream and finally undergoing renal excretion. It is possible that the enterohepatic circle increases the blood half-life of these metabolites, delaying their elimination [98].

In light of the chemical-structural differences of the large family of phenolic compounds, it is clear that their fate within the human organism will be different. Since flavonoids are often found as glycosyl derivatives, their absorption at the small intestine level is associated with the cleavage and release of the aglycone, which can occur through two possible processes: diffusion mediated by the lactase phlorizin hydrolase (LPH) enzyme and transport mediated by cytosolic β-glucosidase (CBG). LPH is present in the brush border of the small intestine epithelial cells, and releases the aglycone, which subsequently enters the epithelial cells through passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane. CBG is found at the epithelial cells level; the aglycone resulting from its action will be transported in epithelial cells with the activation of sodium-dependent glucose transporter-1 (SGLT1) [99]. Before entering the blood stream, aglycones undergo conjugation with glucuronic acid, sulfate and methyl groups, catalyzed by UDP-glucuronosyltransferase (UGT), sulfotransferase (SULTs) and catechol-*O*-methyltransferase (COMT), respectively [99].

However, there are compounds that are not affected by the enzymatic activity of the small intestine, so they arrive intact into the colon where the microbiota can free the aglycones through several reactions, such as ring opening, hydrolysis, demethylation, reduction, decarboxylation, dehydroxylation and isomerization [97].

Chlorogenic acids are hydrolyzed by the esterases of the gut microbiota, releasing a small amount of phenolic acids, which are then absorbed by the intestinal epithelium, therefore being sulfated or methylated before being finally eliminated with the same systems previously described for flavonoids [100]. Anthocyanins are largely and rapidly absorbed

into the gastric lumen, showing high plasmatic levels in a short time. The fact that they can pass into enterocytes as complex derivatives suggests an involvement of certain glucose transporters (for example sodium-glucose transporter 1 – SGLT1 – and glucose transporter 2 – GLUT2). The portion that gets past the stomach without modification and reaches the intestine is hydrolyzed by microflora enzymes, with the release of aglycones, which are then available for liver reactions before their excretion [101].

Beneficial health effects of food polyphenols

In a correct and balanced dietary regimen, polyphenols intake becomes a pivotal factor in the prevention of several pathologies, from various types of cancer [8–10] to chronic and degenerative diseases, that afflict our society such as cardiovascular [11,89], diabetes [12,102], obesity [12] and neurodegenerative diseases [12,102]. Epidemiological studies have shown that polyphenols could act as preventive and/or therapeutic agents, probably due to their antioxidant capacity, which prevents free radicals from forming stabilized chemical adducts, and thus avoiding to trigger chain reactions [102]. In addition, other possible biochemical and molecular mechanisms have been identified, including multiple effects within intra- and intercellular signaling pathways, such as regulation of nuclear transcription factors and lipid metabolism, as well as modulation of inflammatory mediators synthesis, including tumor necrosis factor and interleukins. [103,104].

The incidence of neurodegenerative disorders is increasing worldwide, also due to the increase in the average lifespan. There is currently no definitive cure for any of them, however a proper lifestyle associated with a diet rich in bioactive compounds could have a considerable importance in terms of prevention.
These diseases are characterized by the functional loss of neurons and consequent motor and cognitive activities and two factors that can greatly affect development and progression are neuroinflammation and oxidative cellular imbalance [103]. Given the numerous evidence on antioxidant and anti-inflammatory properties of polyphenols, researchers want to investigate whether these compounds could actually prevent and/or ameliorate the patterns of neuronal deterioration. Numerous epidemiological studies

have shown the ability of polyphenols to act against neurodegenerative diseases, acting through numerous mechanisms, such as interacting in neuronal and glial signaling; modulating cerebrovascular microcirculation, fighting the damages induced by inflammation and neurotoxins [99]. Among dietary polyphenols, anthocyanins showed a marked neuroprotective activity, fighting neuroinflammation, modulating synaptic plasticity and clearance of toxic proteins, with consequent improvement of the cognitive and mnemonic profile. These compounds have also been correlated in the upregulation of the brain-derived neurotrophic factor protein (BDNF) gene, which plays a key role in neuronal growth and differentiation [105]. Other studies have shown that the intake of particularly flavonoid-rich foods (e.g. grape juice, berries and cocoa) had positive effects on cognitive outcome measures on both animals and humans [99].

Just as for neurodegenerative diseases, the battle against cancer is one of the greatest challenges of the contemporary era, as it remains one of the leading causes of death worldwide. For this challenge as well, polyphenols have proved to be valuable allies, both in the nutraceutical and therefore preventive field and as a basis for developing innovative drugs. Dietary polyphenols could be effective in protecting against carcinogenesis, through activities such as modulation of tumoral cell signaling, promotion of apoptosis, modulation of enzymatic activities dedicated to detoxification (glutathione peroxidase, catalase, NADPH-quinone oxidoreductase, glutathione S-transferase and/or cytochrome P450) [106]. Among cancers, pancreatic, hepatic, and biliary ones represent three of the most challenging malignancies facing the oncologist; it is estimated that they are fatal for half of the patients within the first six months [107]. Flavonols, particularly glycosylates ones, have been associated with *in vitro* antiproliferative activity in a dose-dependent manner, and these compounds have also been able to sensitize PANC-1 cancer cell lines to standard antitumor therapy [107]. Similarly, stilbenes have also demonstrated *in vitro* antiproliferative and anticarcinogenic features related to programmed cell death or apoptosis in pancreatic cancer cell lines: PANC-1, BxPC-3, and AsPC-1.

In addition to their pro-apoptotic activity, these compounds have also been investigated for their ability to positively modulate the tumoral extracellular environment, characterized by hyperglycemia and hypoxia; through the mitigation of these conditions by polyphenols, the *in vitro* growth of cancer cell lines has been strongly reduced [107].

It is worth pointing out that most of the findings about the beneficial effect of polyphenols come from *in vitro* studies. It is still not entirely clear whether these mechanisms are fully transposable also *in vivo* [102]. The discrepancies in fact are due to the different concentrations of polyphenols used for the tests on cell lines *in vitro* (range from μM to mM) which, however, decrease significantly *in vivo*, both on humans and animals, reaching concentrations in the range of nanomolar. Other differences are also due to bioavailability issue, already explained in the previous paragraph, and to the high complexity of the system *in vivo*. [99].

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Prunus avium L.

Characterization of new sweet cherry cultivars for their phenolic profile, antioxidant activity and neuroprotective potential

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Article

Fruit Quality Characterization of New Sweet Cherry Cultivars as a Good Source of Bioactive Phenolic Compounds with Antioxidant and Neuroprotective Potential

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Sweet cherry: general information and nutraceutical profile

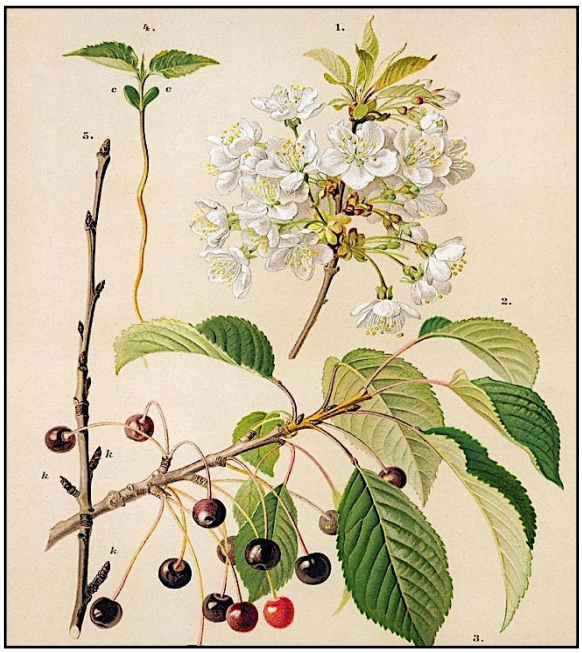

Figure 20: Prunus avium L. *[1]*

Prunus avium L., also known as "wild cherry" or "bird cherry" is a deciduous tree belonging to the Rosaceae family [1]. Despite being native to Caucasus and Balkan regions, this species has been also distributed in other European areas, up to the temperate regions of North Asia and North America, thanks to the diffusion favored by the migration of populations [2]. This tree grows up to 20 meters height, and is characterized by an ovate-pyramidal crown, an early ramified erect trunk, and a brown bark shaded with purple, often marked in horizontal strips. Leaves are alternate, pendulous, simple, and elliptic-ovate or obovate in shape, about 5-15cm x 3-8cm, with serrated margins. Usually they are dull green, glabrous rugose above and sometimes lightly downy. Their pale green color, typical of spring, tends to darken in summer, while in autumn turns yellow, orange-red, scarlet or pink. Flowers are typical of the Rosaceae family; they are perfect flowers with a long petiole, with a green and glabrous calyx, composed of 5 sepals and a corolla formed by 5 white petals demarginated at the apex. Androecium is composed of 15-25 stamina long like petals and yellow anthers; both the ovary and the style are glabrous. Flowers are grouped into corymb inflorescences; flowering usually occurs between April and May. Pollination is entomophilous (mainly by honeybees, wild bees, and bumblebees). Being the wild cherry a self-incompatible species, it needs pollen coming from other plants of the same species for the fertilization. The fruits are roundish fleshy drupes (about 1-3 cm of diameter) of a bright color varying from pink to red-black. The sweet-sour fully ripen epicarp is soft and smooth and is highly appreciated by birds (the Latin specific epithet *avium*, in fact, refers to birds), while the endocarp is woody and hard.

This tree is very valuable also for the semi-porous, discolored wood that is easily polishable and refinable, and for this is sought after not only for furniture and cabinet making, but also for musical instruments and inlay [3]. *Prunus avium* cherry is one of the most loved and appreciated fruits in all temperate areas of the globe. A portion of 100 grams of fresh cherries provides about 38 kcal (158 kJ), and 86% of the fresh mass represents the edible portion. A portion of cherries (corresponding to about 10-12 fruits) is a very low-calorie meal, given the large amount of water possessed by the fruits. Carbohydrates consists mainly of simple sugars (in order of abundance: glucose, fructose, sorbitol and sucrose) which vary depending on the cultivar [4]. Sugars are responsible for the sweet taste of fruits, while tart or sour taste are mainly due to the presence of organic acids (e.g., malic, citric, succinic, lactic, and oxalic acids) which also greatly contribute to the taste [5,6]. Sweetness can be expressed as soluble solids content (SSC) or Brix degree (°Bx), while acidity as titratable acidity (TA) and their ratio (SSC/TA) is considered as an overall taste attribute [7]. The composition in micronutrients, understood as mineral salts and vitamins, is complete and contributes to make these fruits as a good source of potassium, vitamin A and vitamin C, which can help to reach the recommended levels of daily intake. The following table (Tab. 1) summarizes the nutritional values, both for macronutrients and for micronutrients.

Macronutrients (g)		**Micronutrients: mineral salts (mg)**		**Micronutrients: vitamins (mg)**	
Water	86.2	Sodium	3.0	Thiamine (B_1)	0.03
Carbohydrates	9.0	Potassium	229.0	Riboflavin (B_2)	
Proteins	0.8	Iron	0.6	Niacin (B_3)	0.2-0.5
Lipids	0.1	Calcium	30.0	Pantothenic acid (B_5)	0.2
Fiber	1.3	Phosphorus	18.0	Vit A (ret. eq.)	19.0 μg
				Ascorbic acid (vit. C)	7.0-11.0
				Alpha tocopherol (vit. E)	0.1
				Vit. K	2.1 μg

***Table 1**: nutritional profile of a portion of 100 grams of fresh cherries. [8,9]*

Sweet cherries are considered an excellent source of polyphenols, in particular in these fruits we find flavonoids, flavan-3-ols, and flavonols and non-flavonoids such as hydroxycinnamic and hydroxybenzoic acids. The most abundant phenolic compounds are anthocyanins such as cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside, peonidin-3-*O*-rutinoside and glucoside, as well as pelargonidin-3-*O*-rutinoside.

Among the phenolic hydroxycinnamic acids we find the neochlorogenic, p-coumaroylquinic and chlorogenic acids. Finally, among flavonols: quercetin-3-glucoside, quercetin-3-rutinoside and kaempferol-3-rutinoside are found, whereas for flavan-3-ols, catechin and epicatechin are the most representative ones. These compounds are concentrated in peel and pulp of the fruit and undergo fluctuation during ripening. They contribute to the organoleptic and sensory properties of the fruit [7]. Also, the color is a very peculiar and attractive characteristic of cherry fruits, bright and intense red is influenced by anthocyanins content, and it is considered as a marker of ripeness. In fact, in sweet cherry, as well as in other red fruits, during the ripening process the initial green color changes to red, thanks to the accumulation of anthocyanins and the degradation of chlorophyll. These changes can be quantified with three parameters: L* (lightness), and a* and b* (green-red and blue-yellow color components, respectively) [10].

The "Sweet ® cherry": improve the excellence

According to FAOSTAT 2018 estimates, global sweet cherry production has been increasing for at least a decade; in 2018 the largest global producers were Turkey, USA and Uzbekistan, with Italy ranking in the sixth position [11]. The cultivation of sweet cherries in Italy represents a centuries-old tradition, which has developed throughout the country, in areas very different from each other, leading to a significant enrichment in germplasm. This represents an important source of genetic diversity and it is crucial to monitor and preserve it, especially for the breeding programs that will follow, aimed at obtaining new cultivars with improved quality traits [12]. The ancient cultivars of sweet cherry represent an excellent starting point for selecting genotypes with superior traits, both from organoleptic and nutritional points of view. Numerous are the revalued varieties, all peculiar to some Italian territories, as in the case of typical Apulian, Campanian, Emilian and Tuscan cherries. [13].

Sweet cherries are seasonal fruits; their picking period goes from late spring to early summer. The season of these fruits in Europe is quite short compared to America, where several environmental and anthropic factors have managed to extend it by a few weeks [13]. Moreover, cherries have a high tendency to perish; indeed, these fruits can resist, under suitable cooling conditions, only one or two weeks. Together with these difficulties, from the agronomic point of view, there is also the problem of insufficient supply from the market to meet global demand. In light of this, in recent decades, sweet cherries cultivation has undergone radical innovations, which allow to obtain more intensive and specialized orchards.

The choice of cultivars has also recently become of primary importance. It has been focused on cherry accessions that produce fruits of particularly large size (over 12 grams, with a diameter of about 30 millimeters), attractive fruit color (bright or dark red colored fruits are preferred), suitable flesh texture and sugar concentration (> 16° Brix) [14]. Resistance to *cracking*, self-fertilization, and extension of the ripening calendar (especially for early cultivars, whose reference remains the cultivar Burlat) are also considered very important. The wide varietal scenario is nowadays enriched by new varieties derived from breeding programs carried out in different countries of the world, such as Canada, USA, Francia and Italy [14]. The improvement of fruit quality characteristics represents one of the main goals of all these breeding programs. Over the past 20 years, the breeding of sweet cherry has achieved important results in Europe, and more than 200 new varieties have been introduced, especially in Ukraine, Romania, France, and Italy. The most commonly used cherry breeding methodology for obtaining new cultivars is intraspecific crossbreeding followed by selection [15]. Apart from evident qualitative and organoleptic characteristics, the interest of consumers is recently being addressed to the health beneficial properties of cherries. Thus, the challenge of breeders is placed in this perspective of innovation and research, and several breeding programs have been conceived with the aim of releasing on the market new cultivars, possessing these kinds of improved and extended quality attributes. For this purpose, more than thirty years ago the University of Bologna launched a sweet cherry breeding program, by crossing quality-selected European genotypes with self-fertile American counterparts [16], which allowed the release of new cultivars, called Sweet® UNIBO cultivars. These new cultivars have recently obtained European Protected Geographical Indication (PGI) registration.

Aim of the work

It has been widely recognized that phenolic compounds exert a protective effect against oxidative stress [17], and a growing body of preclinical and clinical research has identified various beneficial health effects connected to their intake [18–20]. Oxidative stress is an imbalance between reactive oxygen species (ROS) production and their degradation by the endogenous antioxidant system [21]. ROS accumulation leads to damages to essential biological macromolecules with deleterious consequences [22]. Oxidative stress is considered a common mechanism involved in the onset and progression of the most widespread chronic and degenerative diseases, such as neurodegenerative disorders. The brain is particularly exposed to oxidative stress due to its elevated oxygen consumption, high content of polyunsaturated fatty acids, and low levels of antioxidants [23]. Strong evidence suggests that oxidative stress plays a key role in important neurodegenerative syndromes, such as Parkinson's [24] and Alzheimer's diseases [25]. Neurotrophic factors, such as the brain-derived neurotrophic factor (BDNF), have also been extensively investigated in the context of neurodegeneration [26]. Patients suffering from Parkinson's or Alzheimer's diseases show lower levels of BDNF [27], and this is associated to an increase in degeneration of dopaminergic neurons in the former [28], and to memory impairment in the latter [29]. In this context, the modulation of BDNF has emerged as another promising therapeutic approach to counteract neurodegeneration, and anthocyanins have been suggested as potential modulating agents [30].

The aim of this work is to characterize fruits of seven new sweet cherry cultivars in terms of their quality traits, bioactive compounds, and nutraceutical potential. The most important characteristics from a production point of view were evaluated to assess the commercial attractiveness of the new cultivars. Moreover, profiles of the three main classes of phenolic compounds and their in vitro antioxidant activity were investigated with the aim of selecting the most promising accessions in terms of health-promoting characteristics. In vitro analyses were then carried out to evaluate the potential neuroprotective activity of the selected cultivars in a differentiated neuron-like SH-SY5Y cell line, focusing on their ability to counteract oxidative stress and modulate BDNF expression.

Plant material and sampling procedure

Fruits of sweet cherry (*Prunus avium* L.) cultivars were collected at the full ripeness stage in two consecutive years (2016 and 2017) from an orchard located in Vignola (Emilia-Romagna, Italy). The study included three reference cultivars (Burlat, Grace Star, Lapins), and seven new cultivars released from the UNIBO sweet cherry breeding program (Sweet Aryana® PA1UNIBO*, Sweet Saretta® PA5UNIBO*, Sweet Valina® PA4UNIBO*, Sweet Gabriel® PA3UNIBO*, Sweet Lorenz® PA2UNIBO* and Marysa® PA6UNIBO* grafted on Colt, and Sweet Stephany® PA7UNIBO* on CAB11E). The abbreviated names Aryana, Saretta, Valina, Gabriel, Lorenz, and Stephany (as well as the non-abbreviated name Marysa) will be used from here onward. All cherry trees were grown under similar agronomic techniques (fertilization, irrigation, and pest control). About 250 grams of randomly picked fruits were collected for each of the four different trees of each cultivar. Four pools, corresponding to four biological replicates, were therefore obtained for each cherry variety.

Extraction procedures

After freeze-drying, pits were discarded and the pulp from cherries picked from the same tree was mixed together and finely ground in a knife mill (IKA A11, Staufen, Germany) for 4 × 30 s periods. Pulp powder was then subjected to the "coning and quartering" sampling procedure and two technical replicates were carried out (fig. 21).

Figure 21: freeze-dried sample of cherry pulp (on the left), subsequently subjected to milling (on the right), prior to sampling.

Flavonoids and anthocyanins were extracted from samples following the protocol by Ballistreri et al. [31], with modifications. A 1-g aliquot of freeze-dried powder was extracted twice with 5 mL of a mixture of methanol/HCl (95.5/0.5, V/V) and homogenized (IKA Ultra Turrax T-18 Staufen Germany) for 1 min at speed 5.5. The suspension was sonicated for 20 min at 35°C with an ultrasonic bath (50/60 Hz and 500 W).

After centrifugation (CL10 Thermo Fisher Scientific Carlsbad, CA, USA) for 10 min at 1400×g, the supernatant was transferred into another vial and the sediment was subjected to the second extraction with the same procedure.

The supernatants were merged, mixed, and filtered through Grade 44 (3 µm) ashless filter paper. The final volume of limpid liquid obtained was recorded. The extract was filtered again through a syringe filter (nylon, 0.22 µm pore diameter, from Thermo Fisher Scientific).

Phenolic acids were extracted from samples according to the protocol by Milinović et al.,[32] with modifications. A 1-g aliquot of powder was thoroughly mixed with 20 mL of a mixture of methanol/water (80/20, V/V) and homogenized for 1 min at speed 5.5. After centrifugation for 15 min at 1400×g, the supernatant was filtered through Grade 44 (3 µm) ashless filter paper. The limpid liquid was brought to 25 mL with 85% methanol in a volumetric flask.

HPLC Determination of phenolic compounds

The method for flavonoid analysis was adapted from Milinović et al. [32] Elution was carried out with a mixture of solvent A (water/formic acid 95/5, V/V) and solvent B (acetonitrile), with a composition gradient ranging from 97% to 36% of solvent A and flowing at 0.5 mL/min. Signal at 360 nm was used for quantitative purposes. Recovery values of flavonoids in spiked samples ranged from 79.2 to 91.4 % (RSD < 9.4%, n = 6).

The chromatographic method for the analysis of phenolic acids was carried out as previously described [33]. Gradient elution was carried out with a mixture of solvent A (50 mM phosphate buffer, pH 2.5) and solvent B (acetonitrile) flowing at 0.7 ml/min, going from 97% to 50% (V/V) of solvent A. Signals at 254, 280 and 329 nm were used for analyte quantitation.

Recovery values of phenolic acids in spiked samples ranged from 80.2 to 91.3% (RSD < 10.7%, n = 6). The method for anthocyanin analysis was adapted from Ballistreri et al. [31] Elution was carried out with a mixture of solvent A (water/formic acid/acetonitrile 87/10/3, V/V/V) and solvent B (water/formic acid/acetonitrile 40/10/50, V/V/V), with a composition gradient ranging from 94% to 40% of solvent A and flowing at 1.0 mL/min. Signal at 520 nm was used for quantitative purposes. Recovery values of anthocyanins in spiked samples ranged from 78.6 to 89.3% (RSD < 9.7%, n = 6). The extracts were injected into a Jasco (Tokyo, Japan) HPLC-DAD system, which consisted of a PU-4180 pump, an MD-4015 PDA detector and an AS-4050 autosampler. The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm × 3 mm I.D., 3.5 µm). Injection volume was 20 µL for all determinations.

In vitro antioxidant activity assays

DPPH assays were carried out on phenolic acid sample extracts using a Jasco V-630 double beam spectrophotometer, as described elsewhere. [34] For the setup of calibration curves, 950 µL of 11 µM 2,2'-diphenyl-1-picrylhydrazide (DPPH) in methanol and 50 µL of methanolic Trolox (Tx) solution at different concentrations (0.05-2.0 mM), or 50 µL of methanol (blank solution), were thoroughly mixed in a 2-mL polypropylene vial. For sample analysis, the Trolox solution was replaced with 50 µL of suitably diluted sample. The vial was incubated in the dark at RT for 24 h, then the absorbance of the solution was read at 515 nm. Calibration curves were set up plotting the discoloration ratio as a function of Tx concentration:

$$\frac{ABS\ without\ TX}{ABS\ with\ TX} - 1$$

Then, Trolox equivalents (TE) of the samples were calculated interpolating on the calibration curve.

ORAC assays were carried out on the same extracts (see above) using a Perkin Elmer (Turku, Finland) Viktor X3 multilabel plate reader, as described by Ou et al. [35] The entire assay was conducted in an aqueous medium, specifically using 10mM phosphate buffer (PBS) pH= 7.4. Briefly 210 µL of 10 nM fluorescein disodium solution were added to 35 µL of Trolox (Tx) solution at different concentrations (0-100 µM), or PBS (blank solution), or properly diluted samples, in 96-well microplates and let rest at 37°C for 5 minutes under

shaking. Then 35 µL of 240mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were added and subsequently the microplates were read for 35 times of each sample, 10 seconds apart, at the excitation and emission wavelengths for fluorescein (495 and 550 nm, respectively). Trolox Equivalents (TE) were calculated from the relative area under the curve of the emission intensity vs. time plot.

Cell Culture and Viability

The SH-SY5Y human neuroblastoma cell line was obtained from Merck Italia. Cells were grown in DMEM supplemented with 10% (v/v) of FBS, 2 mM of L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2, as previously reported. [36] Cells were used for experiments after inducing their differentiation with all-trans retinoic acid (10 µM) for 7 days. To test viability, cells were treated with different concentrations (0.1–100 µg/mL) of five cherry extracts (Burlat, Grace Star, Gabriel, Lorenz, Marysa) for 24 h. Oxidative stress was induced with 700 µM H_2O_2 for 1 h. Cell viability was evaluated by measuring MTT reduction.

At the end of the experiments, MTT was added to the cell medium (final concentration 0.5 mg/mL) and incubated for 1h and 30 min at 37 °C. After incubation, MTT solutions were removed, DMSO was added, and the absorbance was measured using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; Perkin-Elmer, Wellesley, MA) at a wavelength of 595 nm.

Intracellular ROS Measurement

The formation of intracellular ROS was evaluated using the fluorescent DCFH-DA probe as previously reported. [37] Briefly, SH-SY5Y cells were treated with 50 µg/mL of each cherry extract for 24 h, then were incubated with 10 µM DCFH-DA in DMEM 1% FBS w/o phenol red for 30 min. After removal of DCFH-DA, cells were incubated with 400 µM H_2O_2 in DMEM 1% FBS w/o phenol red for 15 min. After 15 min H_2O_2 was removed and was replaced by PBS. Cells fluorescence was measured using 485 nm excitation and 535 nm emission with the microplate spectrofluorometer (VICTOR3 V Multilabel Counter).

Determination of Reduced Glutathione (GSH) Levels

Reduced glutathione (GSH) levels were determined using the MCB fluorometric assay as previously reported [38]. Briefly, SH-SY5Y cells were treated with 50 µg/mL of each cherry extract for 24 h before the induction of oxidative stress with 700 µM H2O2 for 1 h. After treatment, the cells were incubated with 50 µM MCB 1% FBS w/o phenol red for 30 min at 37°C. After incubation, fluorescence was measured at 355 nm (excitation) and 460 nm (emission) with a microplate spectrofluorometer.

Analysis of mRNA expression

SH-SY5Y cells were treated with 50 µg/mL of each cherry extract for 24 h and, after treatment, total RNA was extracted using RNeasy Mini Kit, following the manufacturer's protocol. The yield and purity of RNA were measured using a NanoVue spectrophotometer (GE Healthcare, Milano, Italy). mRNA was reverse-transcribed into cDNA starting from 1 µg of total RNA using iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. The subsequent polymerase chain reaction (PCR) was performed in a total volume of 10 µL containing 2.5 µL (12.5 ng) of cDNA, 5 µL SsoAdvanced Universal SYBR Green Supermix (BIO-RAD) and 0.5 µL (250 nM) of each primer.

Primers used were: BDNF 5'CAAAAGTGGAGAACATTTGC3' (forward) 5'AACTCCAGTCAATAGGTCAG3' (reverse), glutathione reductase (GR) 5'GACCTATTCAACGAGCTTTAC3' (forward) 5'CAACCACCTTTTCTTCCTTG3' (reverse), NAD(P)H quinone oxidoreductase (NQO1) 5'AGTATCCACAATAGCTGACG3' (forward) 5'TTTGTGGGTCTGTAGAAATG3' (reverse), ribosomal protein S18 (RPS18) 5'CAGAAGGATGTAAAGGATGG3' (forward) 5'TATTTCTTCTTGGACACACC3' (reverse) from Merck Italia. RPS18 was used as a reference gene. cDNA amplification was started by activating the polymerase for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. A melt curve was run to ensure quality control and generation of a single product. Normalized expression levels were calculated relative to control cells according to the $2^{-\Delta\Delta C_T}$ method. [39]

Statistical analysis

Each experiment was performed at least three times, and all values are represented as mean ± SE, which was calculated on the four biological replicates of the four samples. One-way ANOVA was used to compare differences among groups, followed by Tukey's test or Dunnett's test (Prism 6; GraphPad Prism Software, San Diego, CA). Values of $p < 0.05$ were considered statistically significant. Correlation analysis was carried out with the same software. A canonical discriminant analysis of some collected data (ORAC values, anthocyanin, phenolic acid and flavonol contents) was performed with the library 'candisc' of the R software (3.6.1 version).

The selected set of cultivars uniformly covers the sweet cherry harvesting season in the Emilia-Romagna region, starting from Burlat (May 23rd) and ending with Stephany (June 17th). The following fruit characteristics were analyzed: diameter, weight and pulp firmness, kernel weight, content of soluble solids, sugars and organic acids, titratable acidity, as well as fruit color parameters. The complete fruit quality description of each cultivar is reported in Antognoni et al. (2020) [40].

Phenolic compounds profile

The characterization of the new sweet cherry cultivars for their phenolic profile was carried out for two consecutive years (2016 and 2017) and compared to that of three reference commercial cultivar (as described in materials and methods). Although the extraction and analyses were carried out from dried material, results are expressed on fresh weight basis, in order to compare data with those present in literature. The main classes of phenolic compounds in the pulp of sweet cherry are anthocyanins, phenolic acids (hydroxycinnamic acid derivatives), and flavonols, thus HPLC-DAD analyses were carried out to identify and quantify compounds belonging to these three classes of secondary metabolites.

Anthocyanins

From the chromatographic analysis of sweet cherry extracts enriched in anthocyanins, four compounds have been identified and quantified in all cultivars (in order of abundance): cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside, peonidin 3-*O*-rutinoside and peonidin 3-*O*-glucoside.

In Figure 22 (A, B) the profile of **cyanidin 3-*O*-rutinoside** in cherry fruits is shown, during the two years of investigation. Its concentrations ranged from 50 to 300 mg for 100 grams of fresh weight. In the first year, Gabriel, Lorenz and Aryana turned to be the richest UNIBO cultivars in cyanidin 3-*O*-rutinoside and, among them, Gabriel showed levels about two-fold higher than all three commercial cultivars, while those of Lorenz and Aryana were higher compared to Burlat and Grace Star, but not to Lapins.

All other UNIBO genotypes showed cyanidin-rutinoside levels rather similar among each other, similar to Grace Star and Lapins, and significantly higher than Burlat. In the second year, Gabriel was confirmed to be the UNIBO genotype with the highest level, reaching levels about three-fold higher than the commercial cultivars, followed by Saretta; Lorenz and Aryana had a lower content than the previous year, rather close to all the other genotypes. Differences were also observed from the first and the second year as concerns reference cultivars, with Burlat and Lapins showing an evident decrease in cyanidin 3-*O*-rutinoside content in the latter compared to the former.

The second most abundant anthocyanin was **cyanidin 3-*O*-glucoside**. The profile of this compound in fruits of all cherry cultivars during the two years of analysis is shown in Figure 22 (C, D). The concentrations of cyanidin-glucoside ranged from a minimum of 1 mg to a maximum of 50 mg per 100 grams of fresh cherries. The highest value, in the first harvest year, were found in Burlat, which showed a concentration about seven-fold higher than the other reference genotypes. Among UNIBO cultivars, Aryana showed the highest level, while all the others did not significantly differ among each other and compared to Grace Star and Lapins. In the second year, a general decrease in cyanidin 3-*O*-glucoside levels occurred in all genotypes: Burlat was confirmed as the richest cultivar, while among UNIBO ones, Gabriel turned the to be significantly higher than Grace Star and Lapins. All other cultivars had levels very close among each other and to latter two reference cultivars.

Peonidin 3-*O*-rutinoside was the third anthocyanin in order of abundance, found in cherries extracts. Its content varied among cultivars, from a minimum of 1 mg up to a maximum of 25 mg in 100 grams of fresh cherries. Figure 22E and 22F show the pattern of this compound in all analyzed cherries, In both harvest years. Cultivars can be divided into three groups, based on its levels: in the first year the richest cultivar was Stephany, with more than 20 mg/100 g FW, followed by Lorenz, Gabriel, Saretta, Aryana, and Lapins, with levels ranging from 10 to 20 mg/100 g FW); the group with the lowest levels (less than 10 mg/100 g FW) was represented by Valina, Marysa, Grace Star and Burlat. In the second year, Stephany was confirmed as the richest genotype, followed by Gabriel and Saretta, while Aryana, Lorenz and Lapins showed a decrease compared to the previous year.

Peonidin 3-*O*-glucoside was the least abundant anthocyanin detected in our cherry samples, Its concentrations being always below 1 mg per 100 grams of fresh weight. From the figure 2G it can be noted that for all the cherries belonging to the first year of harvest the profile was quite similar, a part for Aryana, which was the only cultivar significantly different from all the others. The pattern of the second year of collection (figure 22 H) shows much differences among cultivars, with Gabriel as the genotype with the highest content of peonidine 3-*O*-glucoside.

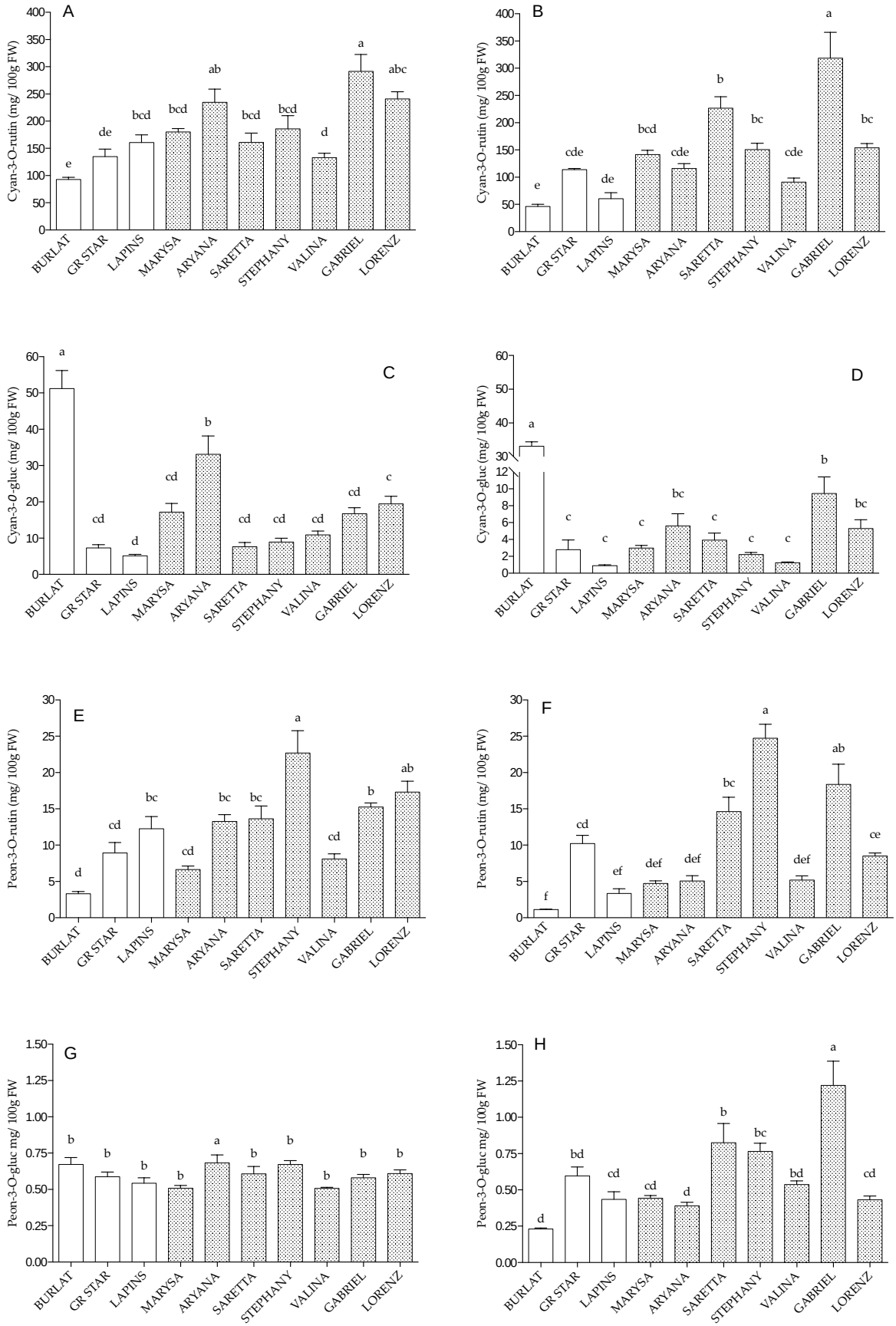

Figure 22: Cyanidin 3-O-rutinoside (A,B), cyanidin 3-O-glucoside (C,D), peonidin 3-O-rutinoside (E,F) and peonidin 3-O-glucoside levels (G,H) (mg/100 g FW) in fruits of sweet cherry cultivars harvested in 2016 (left column) and 2017 (right column). Data are the mean ± SE, different letters indicate statistical significance ($p < 0.05$).

Phenolic acids

From the chromatographic analysis of the sweet cherry extracts enriched in phenolic compounds, three hydroxycinnamic acid derivatives have been identified and quantified in all cultivars: neochlorogenic acid, coumaroylquinic acid and chlorogenic acid. Phenolic acid profiles are shown in fig. 23. In all genotypes, neochlorogenic acid was the main hydroxycinnamic acid derivative found, followed by coumaroyl-quinic and chlorogenic acids, respectively.

Neochlorogenic acid levels ranged from 15 to 65 mg/100 g FW (Fig. 23 A, B). Within this range, three groups could be clearly defined in the first harvest year: one group, comprising Marysa and Lapins, with levels higher than 50 mg/100 g FW: an intermediate group, comprising Grace Star, Saretta, and Stephany, with levels ranging from 30 and 50 mg/100 g FW; a third group, comprising Burlat, Aryana, Valina, Gabriel, Lorenz, with levels below 30 mg/100 g FW. In the second year, the differences in neochlorogenic acid levels among genotypes were more attenuated than in the previous year, even though the pattern was rather similar. Marysa was confirmed as the UNIBO cultivar with the highest neochlorogenic acid content, which turned significantly higher compared to all the other genotypes (Fig. 23 A, B).

Coumaroylquinic acid content ranged from 1.5 to 17 mg/100g FW. In the first year, Lorenz had the highest and Marysa the lowest coumaroylquinic acid levels (Fig. 23 C). In the second years, among UNIBO cultivars, Marysa was confirmed to have the lowest coumaroylquinic acid content, while Lorenz showed a slight decrease compared to the previous year (Fig. 23 D).

As concerns chlorogenic acid, its levels range from 2 to 4 mg / 100 g FW in all genotypes (Fig. 23 C). and only slight fluctuations were observed both within UNIBO and reference cultivars in the two years of investigation (Fig. 23 F).

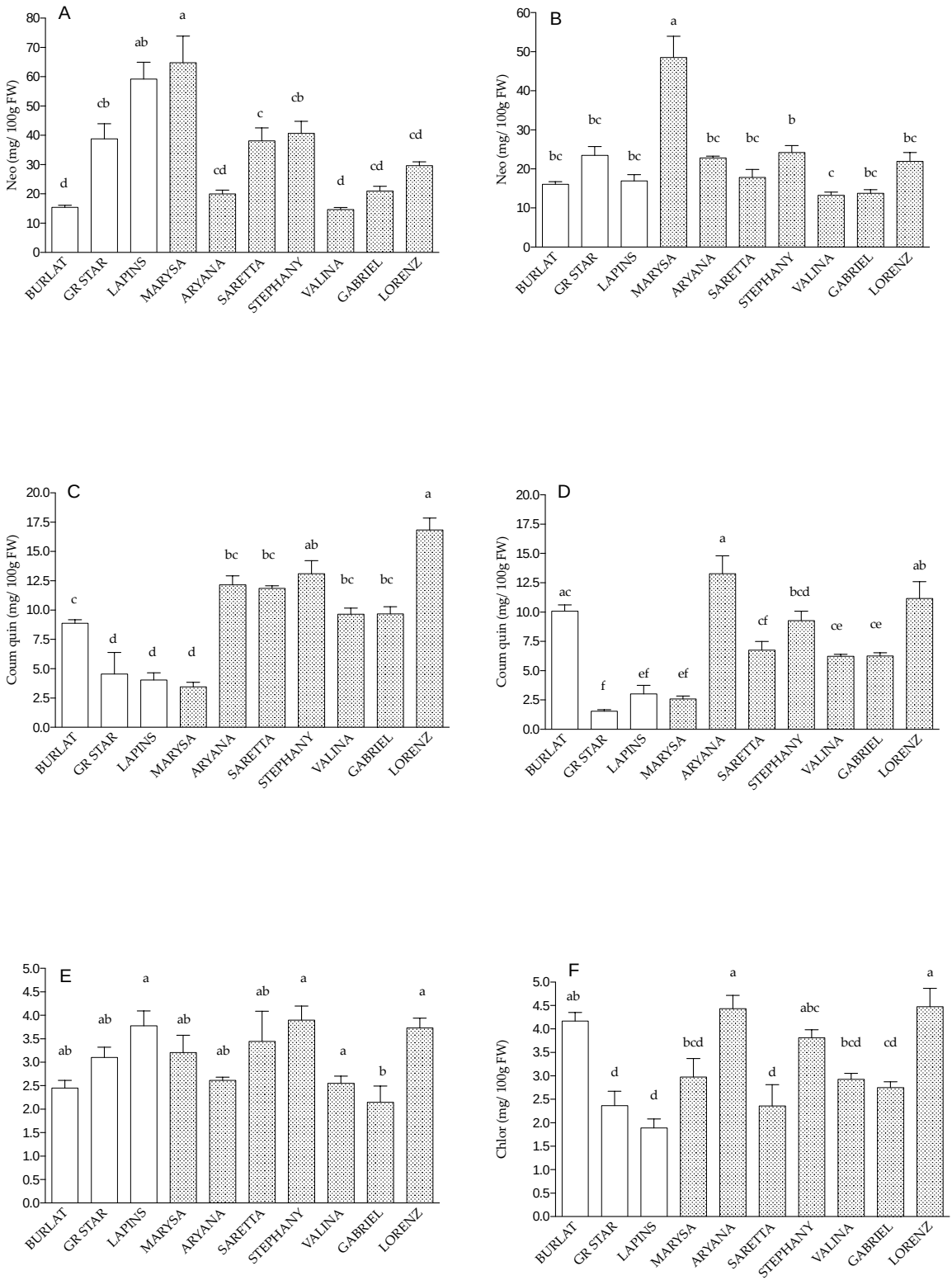

Figure 23: Neochlorogenic acid (A,B), coumaroylquinic acid (C,D), chlorogenic acid (E,F) (mg/100 g FW) in sweet cherry cultivars harvested in 2016 (left column) and 2017 (right column). Data are the mean ± SE. Different letters indicate statistical significance ($p < 0.05$).

Flavonoids

From the chromatographic analysis of the sweet cherry extracts enriched in phenolic compounds, three glycosylated flavonols have been identified and quantified in all cultivars (in order of abundance): **quercetin 3-*O*-rutinoside** (or rutin), **kaempferol 3-*O*-rutinoside** and **quercetin 3-*O*-glucoside**.

Figure 24A shows the profile of the most abundant compound, quercetin 3-*O*-rutinoside (or rutin), in all analyzed cultivars in the first year of harvest, while in figure 24B results of the second year of harvest are shown. In the first year of harvest, reference cultivars had rutin levels between 2.5 and 4 mg/100g FW, and, among UNIBO genotypes, Lorenz and Gabriel showed a content almost double compared to them. The latter was confirmed as the richest in rutin also in the second year, while for Lorenz a decrease in this quercetin derivative occurred. In this harvest year, Saretta also showed a rutin content significantly higher than all reference cultivars, and very similar to that of the previous year. As regards kaempferol 3-*O*-rutinoside, its levels ranged from 0.1 to 1.25 mg/100g FW (fig. 24 C,D). In the first year, all new cultivars showed rather similar concentrations of this kaemferol derivative, and only Saretta had a higher level than Burlat, but not different compared to the other two reference cultivars (fig. 24C). In the second year, the amount of this compound (fig. 24D) was in the range 0.1-0.6mg/100g FW, the richest cultivar was Saretta, although not significantly different from all others, while the poorest was Aryana. A similar pattern was found for quercetin 3-*O*-glucoside. In fact, in fruits harvested in 2017 (fig. 24 F), the values were lower compared to those of the previous year (0.01-0.4 vs 0.1-1.0 mg/100g FW, as shown in fig. 24E). Reference cultivar Burlat maintained the highest concentration of this compound in both years of analysis, while the pattern for UNIBO genotypes was somewhat variable.

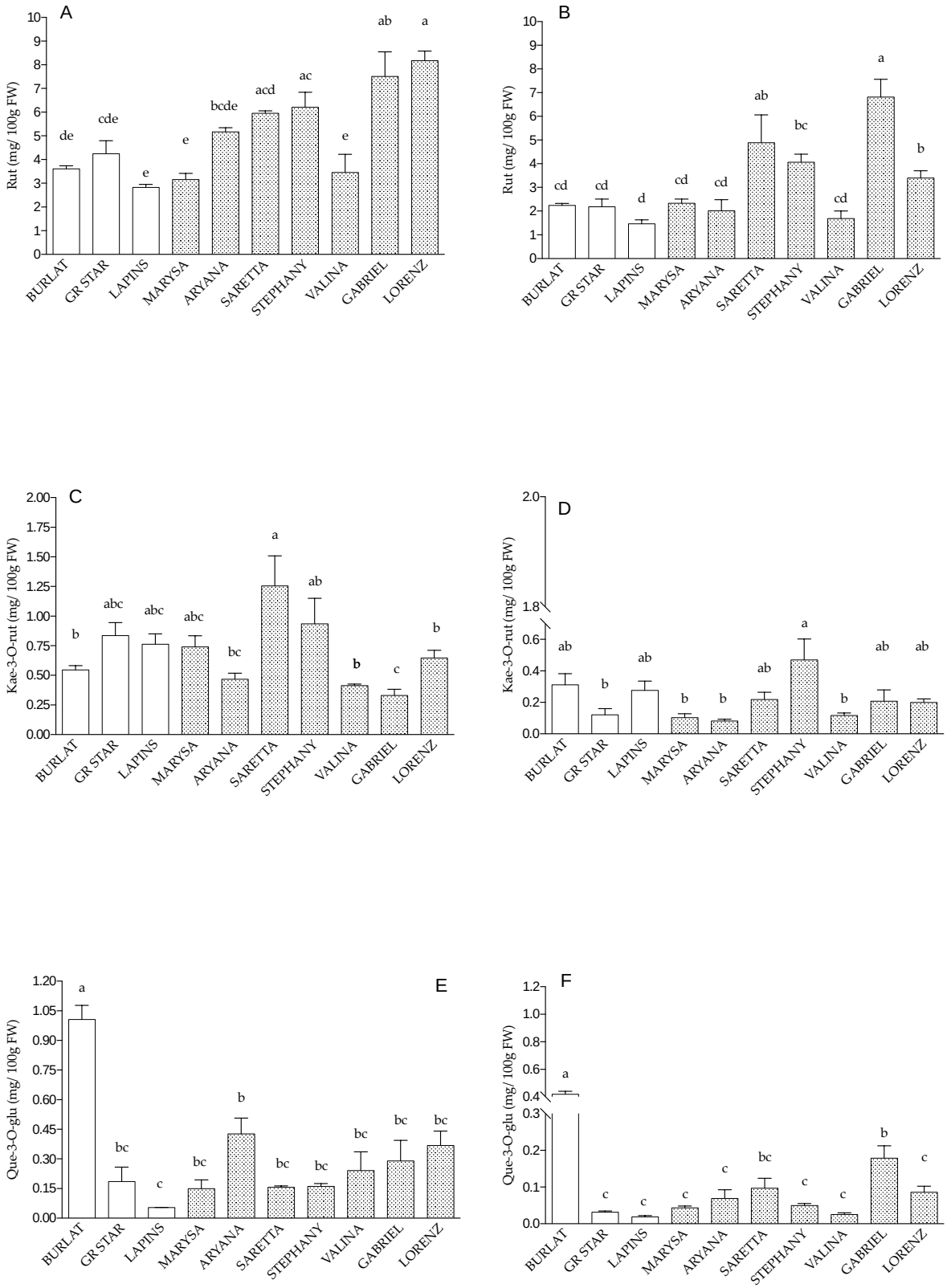

Figure 24: Quercetin 3-O-rutinoside or rutin (A,B), kaempferol 3-O-rutinoside (C,D), quercetin 3-O-glucoside (E,F) (mg/100 g FW) in sweet cherry cultivars harvested in 2016 (left column) and 2017 (right column). Data are the mean ± SE. Different letters indicate statistical significance ($p < 0.05$).

The total index of each class of phenolic compounds found by chromatographic analysis was calculated by summing up the individual concentrations of each compound over the two years of analysis. The pattern of total anthocyanin index (TAI), total phenolic acid index (TPAI) and total flavonoid index (TFI) in cherries of all cultivars are shown in figure 25, and it reflects the pattern of the major components of each class of phenolics. From the graphs it is possible to better visualize how these compounds changed, in quantitative terms, in the two years of harvest.

As concerns TAI (fig. 25A), there was a general decrease in 2017, which turned statistically significant in some cases. Among the reference cultivars, Burlat and Lapins reported a halving of the TAI value, while reference Grace Star maintained more stable anthocyanin levels. Among UNIBO genotypes, Gabriel showed a rather stable concentration of anthocyanins in both years of analysis, together with Saretta.

As for the TPAI value reported in figure 25B, cultivars that maintained a stable content of phenolic acids during the two years of analysis were Burlat for the reference cultivars, and Marysa, Aryana and Valina for UNIBO cherries. For the remaining cultivars, the second year saw a more or less marked decrease in TPAI: for example, Lapins' TPAI was more than halved, as well as Saretta's.

The TFI is shown in figure 25C, from which it can be seen that the only stable varieties in terms of flavonoid concentration were the UNIBO Saretta, Valina and Gabriel genotypes. All the others, both reference ones and the remaining UNIBO cultivars, showed a significant decrease in the second year of analysis. In the cases of Grace Star, Lapins, Aryana and Lorenz the difference was quite evident, having half the content recorded in 2016.

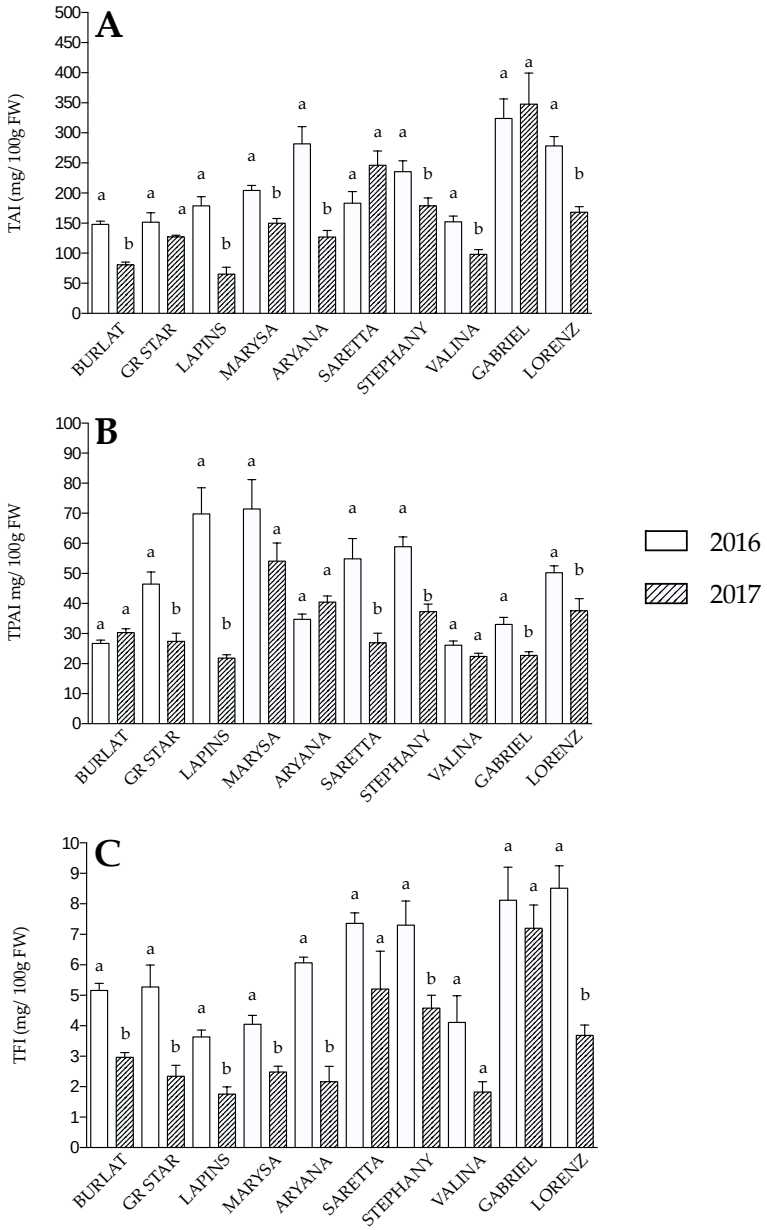

*Figure 25: Total Anthocyanin Index, TAI (A); Total Phenolic Acid Index, TPAI (B); Total Flavonoid Index TFI (C) in sweet cherry cultivars in the two years of investigation 2016 and 2017. Data are the mean ± SE. Different letters **within cultivar** indicate statistical significance ($p < 0.05$).*

In vitro antioxidant activity

The antioxidant activity of sweet cherry extracts was measured through two different assays, DPPH and ORAC. The results of the DPPH assay (fig. 26 A,B) show immediately that the pattern of antioxidant activity of the two years was fairly homogeneous, the values in fact range from 2.5 to 10 mmol TEQ/100g FW.

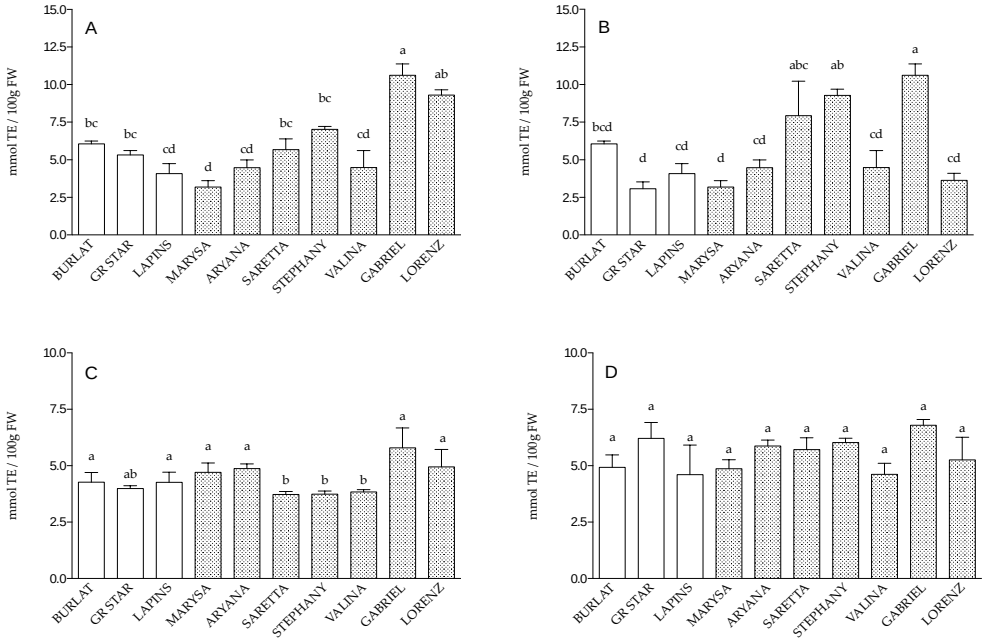

Figure 26: Antioxidant activity measured with DPPH (A, B) and ORAC (C, D) assays (mmol TEQ/100g FW) in sweet cherries cultivar harvested in two consecutive years: 2016 (left column) and 2017(right column). Data are the mean ± SE of four biological replicates. Different letters indicate statistical significance ($p < 0.05$).

The highest activity was found in Gabriel, reaching 10 mmol TEQ/100g FW, while the lowest values was recorded in Marysa. As concerns results of the antioxidant activity measured by the ORAC assay, differences among genotypes were much less pronounced in comparison to those of DPPH, and no significant differences of UNIBO genotypes compared to commercial ones were observed (Fig. 26 C,D). Antioxidant activity was found to be mainly statistically correlated with TAI and TFI, rather than with TPAI. In detail, a positive correlation was found between TAI and antioxidant activity measured by DPPH and ORAC assays (r Pearson coefficients 0.655 and 0.793 respectively), which was also confirmed in the second year of analysis (r Pearson coefficients: 0.739 and 0.512). An additional positive correlation was recorded between TFI and DPPH assay results obtained in 2016 and 2017 (r Pearson coefficients: 0.856 and 0.847 respectively).

Results of phenolic compounds and antioxidant capacity obtained from the first year of analysis were evaluated through principal component analysis (**PCA**), in order to select the most diverse cultivars, to be subjected to biological activity assays. This is due to the fact that second year analysis had the primary objective of confirming the stability of the genetic modification and understanding the possible influence of seasonality on the phytochemical profile of the fruits. Figure 27 shows the results of PCA of antioxidant activity (ORAC) and anthocyanin, phenolic acid, and flavonol content. Around 73% (46.2 and 26.2) of the observed variability is explained by the first two components. The first component is significantly positively correlated with the content of neochlorogenic acid, while the second component is correlated with the content of peonidine 3-*O*-rutinoside and cyanidine 3-*O*-rutinoside.

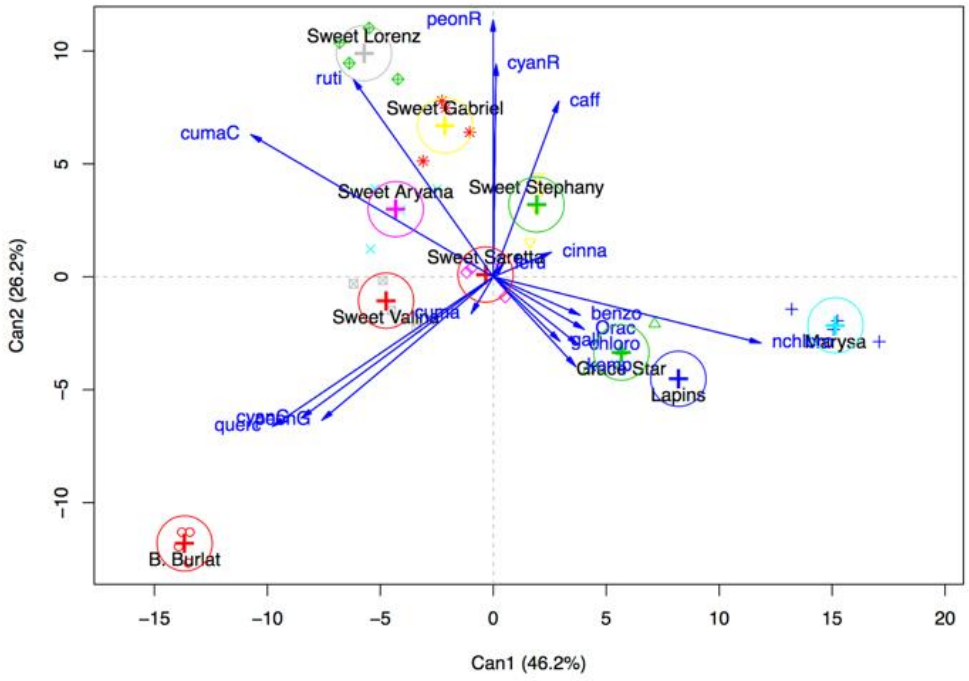

Figure 27: Canonical discriminant analysis of the antioxidant activity and anthocyanin, phenolic acid, and flavonol contents of sweet cherry extracts. Can 1 = canonical component 1; Can 2 = canonical component 2.

Based on this exploratory analysis, two reference cultivars (Burlat and Grace Star) and three UNIBO cultivars (Gabriel, Lorenz and Marysa) resulted to be very diverse in terms of phenolic compound content and were selected for the subsequent assessment of the protective effects of cherry extracts.

Neuroprotective effect of cherry extracts against oxidative stress

Since several studies have demonstrated that the classes of polyphenols present in new cherries possess neuroprotective activity [41,42], the extracts were evaluated for their beneficial effects against oxidative stress in neuron-like differentiated SH-SY5Y cells. SH-SY5Y cells are widely used as an *in vitro* model of neuronal function [43] and their exposure to H_2O_2 triggers metabolic modifications leading to cell death and mimicking the events that might occur during neurodegeneration [44]. First of all, the cytotoxicity of cherry extracts was evaluated with the MTT assay using different concentrations in the 0.1-100 µg/mL range for 24 h. None of the concentrations tested showed cytotoxicity to cells. Subsequently, the protective potential of cherry extracts was tested by using them as a pretreatment (at the concentration of 0.1-100 µg/mL of each extract) on cells for 24 h before exposing them to H_2O_2 for 1 h to induce oxidative stress as reported [36]. Genotypes Gabriel, Lorenz, Grace Star and Marysa significantly increased viability in cells exposed to H_2O_2, as reported in fig. 28. Gabriel was the most effective one, with significant protection at all concentrations higher than 1 µg/mL, while Burlat did not show any protection against oxidative stress.

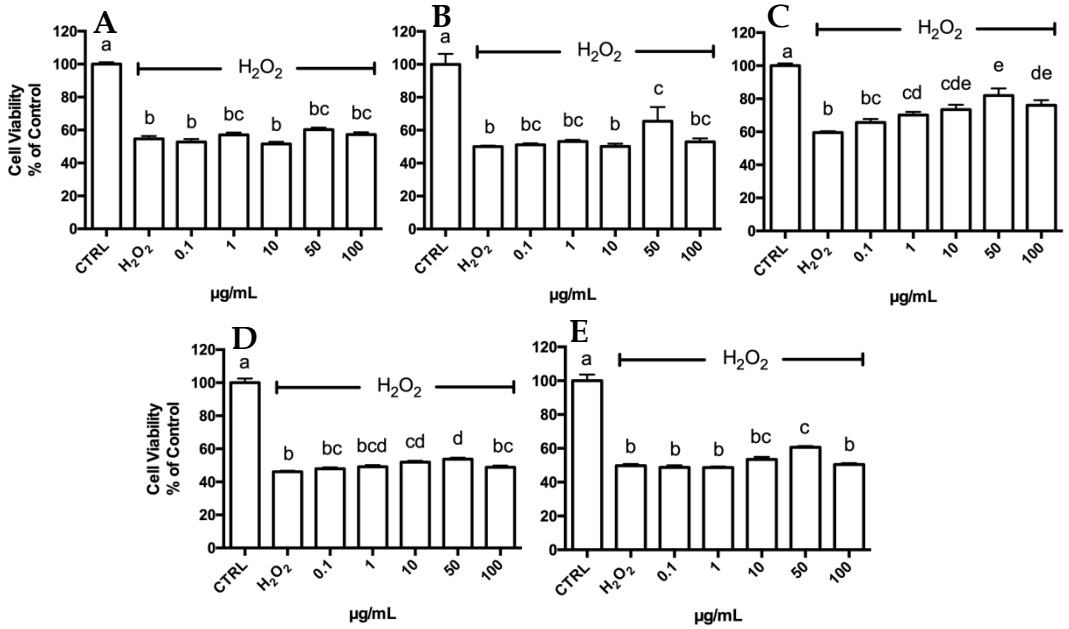

Figure 28: Protective effect of cherry extracts Burlat (A), Grace Star (B), Gabriel (C), Lorenz (D), Marysa (E) against H2O2-induced damage (700 µM) to SH-SY5Y cells. Each bar represents the mean ± SEM of at least three independent cell viability experiments. Different letters indicate statistical significance ($p < 0.05$).

As 50 µg/mL was the only concentration that led to significant protection in most extracts, this concentration was used for subsequent experiments.

The ability of the cherry extracts to act against H_2O_2-induced intracellular ROS production was further investigated by the DCFH-DA assay. In agreement with results of viability, genotypes Gabriel, Grace Star, Marysa, and Lorenz led to a significant reduction in intracellular ROS levels in cells exposed to H_2O_2, while Burlat did not produce any significant effect (fig. 29 A). Moreover, the effect of the cherry extracts on intracellular GSH levels was evaluated by the MCB assay, since is the most abundant intracellular antioxidant. SH-SY5Y cells were treated with 50 µg/mL of each extract for 24 h and then exposed to H_2O_2. As expected, H_2O_2 treatment led to significant reduction of GSH levels in respect to control cells. Only Grace Star and Gabriel extracts significantly increased GSH levels when compared to H_2O_2-exposed cells, up to values comparable to those of control cells. (fig. 29 B).

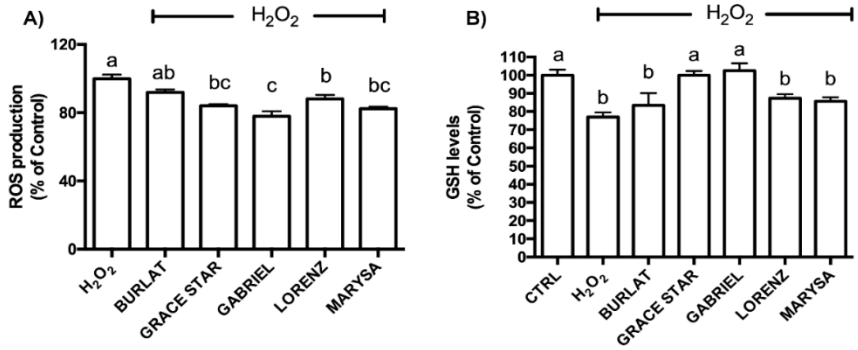

Figure 29: Effects of cherry extracts on intracellular (A) ROS and (B) GSH levels in SH-SY5Y cells. ROS data are expressed as a percentage compared to H_2O_2-treated cells, GSH levels are expressed as a percentage compared to control (CTRL) cells; each bar represents the mean ± SEM of at least three independent experiments. Different letters indicate statistical significance ($p < 0.05$).

Interestingly, a positive correlation (r Pearson coefficients: 0.89), was found between GR expression and total anthocyanins index (TAI).

Effect of cherry extracts on antioxidant enzyme and BDNF expression

Since antioxidant capacity of cherry extracts could also occur through the modulation of the endogenous antioxidant system, the expression of two endogenous antioxidant enzymes: glutathione reductase (GR) and NAD(P)H dehydrogenase [quinone 1] (NQO1), was analyzed by real-time PCR. Cells were treated with 50 µg/mL of the five extracts before measuring changes in mRNA expression.

Genotypes Grace Star, Gabriel, Lorenz and Marysa significantly upregulated both enzymes, while Burlat did not influence the expression of these enzymes (fig. 30). Gabriel was the most effective one in upregulating GR.

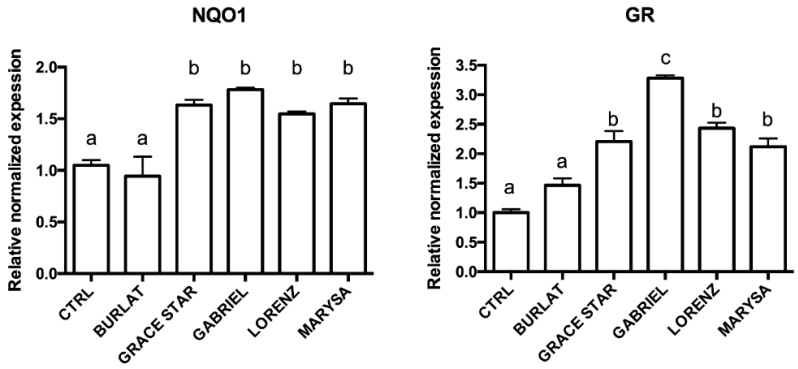

Figure 30: Effect of cherry extracts on the expression of NQO1 and GR in SH-SY5Y cells. Each bar represents the mean ± SEM of at least three independent experiments. Different letters indicate statistical significance ($p < 0.05$).

Given the key role of BDNF in neurodegenerative diseases, the effect of the different extracts on Brain-derived neurotrophic factor (BDNF) expression levels was also investigated. Cells were treated with 50 μg/mL of the extracts before measuring changes in BDNF mRNA expression. All cultivars were able to significantly up-regulate BDNF when compared to control cells as reported in fig. 31, suggesting a protective role of cherries in neurodegeneration, possibly through a mechanism other than the antioxidant activity.

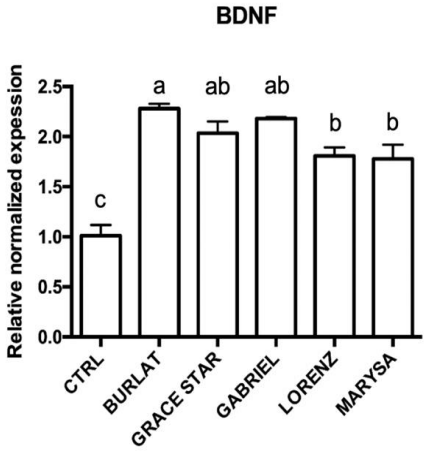

Figure 31: Effect of cherries extracts on the expression of BDNF in SH-SY5Y cells. Each bar represents the mean ± SEM of at least three independent experiments. Different letters indicate statistical significance ($p < 0.05$).

Results here obtained show that the cherry cultivars clearly differ from each other in fruit quality parameters and in health-promoting compound content. Indeed, the pattern of polyphenolic compounds known to characterize *Prunus avium* fruits showed significant quantitative differences among cultivars. Results are in line with most of the results reported in literature, in fact compounds cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside, and peonidin 3-*O*-rutinoside are found to be the majority while peonidin 3-*O*-glucoside is identified as a minor compound. [45–47] For cyanidin 3-*O*-rutinoside, our results are in line with those reported by Nawirska-Olszańska et al. [45] in ancient cherry cultivars grown in the Czech Republic, but are significantly higher than those reported by other authors [4,31,48]. On the other hand, the amounts of cyanidin 3-*O*-glucoside are comparable to those reported by Ballistreri et al. [31], since Burlat is the richest cultivar in keracyanine (34 mg/100g FW), while in Grace Star is the poorest (with only 2 mg/100g FW). Other authors report very variable concentrations depending on the cultivars analyzed [49–51] and this could stem, in addition to the intrinsic biological variety of each cultivar, also from the harvest period and fruit storage temperatures [52]. The results obtained from the analysis of glycosylated peonidine are also in agreement with the literature, however peonidine 3-*O*-glucoside is not often identified because of its low concentration [53].

The qualitative profile of hydroxycinnamic acids, identified and quantified in the extracts of sweet cherries of each cultivar, is consistent with what is reported in the literature, where neoclorogenic, chlorogenic and cumaroylquinic acid are the majority components. [54,55] In all analyzed cultivars, neochlorogenic acid represented the predominant compound, confirming what has been reported by other authors [4,56–58]. Even the concentrations of these compounds we found, are in agreement with what is present in literature [31,52,57], however in some cases discrepancies are found. UNIBO cherries were richer in neoclorogenic acid and chlorogenic acid, compared with results reported Milinović (2016) and Jakobek (2009) [32,50], but conversely our quantification of these phenolic acids were lower, compared to those found by others studies [51,59].

Neverthless, these quantitative fluctuations were expected, since they are related to multiple factors, including growing conditions, ripeness stage, post-harvest storage, sample preparations, extraction procedures, and analytical techniques [6]. Glycosylated flavonols, in particular quercetin derivatives, are among the most common compounds found in sweet cherries [4,50]. According to our results, rutin is the majority compound, followed by quercetin 3-*O*-glucoside and kaempferol 3-*O*-rutinoside, and is in line with the literature [47,60].

With regard to the concentration of rutin, our results are comparable to what was reported by Jakobek (2009) [50] on some Croatian cultivars, and some Italian varieties analyzed by Ceccarelli (2018) [51], while they are lower than those reported by Nawirska-Olszanska (2017) [45], which has an average value of about 19mg/100g FW, about twice ours. Quercetin 3-*O*-glucoside is often identified as a minority compound in cherries, as confirmed by Commisso (2017) [57]; in addition, even in their case, cultivar Burlat were the richest, with a fluctuation in the two years of analysis completely comparable to what we reported. Higher values were reported by Martini (2017) [53], exceeding 20 mg/100g FW, in particular Lapins showed levels of 4.28 mg/100g FW, while our results showed only trace amounts. As for the results of kaempferol 3-*O*-rutinoside, they are in line with what was reported by Crupi (2014) [60] on Italian cultivar Ferrovia, with an average of about 0.5 mg/100g FW. Once again, it is fair to say that flavonols variability, in sweet cherry extracts, is strongly influenced by the genotype, as well as by environmental factors and sample treatment [6]. The biosynthesis and accumulation of phenolic compounds is closely related to endogenous and exogenous factors. The former obviously relate to genotype and to the physiological conditions of the plant, whereas the latter include all environmental stimuli, biotic and abiotic the plant is subjected to during its life cycle (i.e., light and water availability, soil composition, temperature and interaction with pathogens and parasites). All cherry fruits we analyzed have been harvested at full maturity, thus minimizing fluctuations due to harvesting at mid-ripeness, which are generally related to a lower quantity of phenolic compounds [52]. The influence of climatic conditions on the quantitative profile of anthocyanins and colorless polyphenols in sweet cherry fruits has been observed by other authors [52]: it has to be underlined that climatic conditions during the spring-summer season were very different in 2017 compared to 2016, with

higher temperatures (25.3 °C vs 22.3 in June, and 18.9 °C vs 17.7 °C in May, respectively) and a longer drought in the former compared to the latter.

It is known that different temperature conditions induce quantitative and/or qualitative changes in the anthocyanin profile in different fruits [61], and that increasing temperatures tend to decrease anthocyanin content [62,63]. Vuletić (2017) analyzed fluctuations of phenolic compounds in cultivars of sour cherries grown in two different places and for three consecutive years and the results show that, despite climatic year and orchard localization being determining factors, what most affects polyphenols content is undoubtedly the genotype [64]. The same considerations have been reported by other studies, on other fruits on different harvest years: the influence of climatic and agronomic conditions is one of the key factors which needs to be closely monitored if the aim is to optimize polyphenol concentrations in full ripeness fruits [65,66].

As concerns our results of antioxidant capacity of cherries extracts, slightly lower results were reported by Ballistreri (2013) [31] and Serra (2011) [46], who reported a range of antioxidant capacity (measured with the ORAC assay) from 0.64 to 3.166 mmol TE/100 g FW and from 1.32 to 3.54 mmol TE/100 g FW, respectively. Results of antioxidant activity on sweet cherry extracts measured by the two assays, DPPH and ORAC, were not univocal, in fact only the DPPH assay showed differences among genotypes, while the results of the ORAC assay were more homogeneous. However, this is due to the fact that the two tests do not share the same mechanism of action. Two mechanisms are at work during the deactivation of a free radical by an antioxidant: one is based on the transfer of a hydrogen atom (HAT mechanism), and the second one on the transfer of a single electron (ET mechanism). The ORAC assay belongs to the first group, since it measures the antioxidant inhibition of oxidations induced by peroxyl radicals and therefore reflects the antioxidant activity of radical chain-breaking by transfer of hydrogen atoms. The DPPH assay, on the other hand, is more oriented towards the second mechanism of action, as it is based on the measure of reducing ability against the radical DPPH• [67]. From a biological point of view, the ORAC assay is certainly more realistic, both because it uses peroxyl radicals and because it takes place in in a physiological-like environment. On the other hand, the DPPH assay is certainly faster and simpler. However, due to the complexity of

the antioxidant issues, there are many factors that if not properly controlled, would affect the outcome.

Indeed, the antioxidant capacity of foods depends on many factors, including the colloidal properties of the substrates, the conditions and stages of oxidation, and the localization of antioxidants in different phases. Moreover, crude plant extracts are very complex mixtures of several components, having different inhibition kinetics [68]. In view of this, it is clear that there will be some discrepancies in the results of such different tests, however it is preferable to apply several of them, based on different mechanisms, to better clarify the antioxidant capacity of samples [69].

The classes of compounds identified in the new cherry cultivars have been reported to exhibit neuroprotective activity [41,42]. Thus, we evaluated the protective effect of the five most diverse cultivars (as assessed by PCA analysis) against oxidative stress in neuron-like differentiated SH-SY5Y cells. These cells, which are generated from neuroblastoma, are widely used as an *in vitro* model of neuronal function [43], moreover they are frequently stimulated to differentiate by *trans*-retinoic acid to achieve more neuron-like features, including neurite expansion and morphological modifications, in order to mimic neuron responses in studies [70].

Treatment with extracts of cherry cultivars protected cells against the oxidative stress induced by H_2O_2, and this result was associated to a significant reduction in intracellular ROS and a significant increase in GSH levels. The Gabriel genotype was confirmed as the most promising and effective cultivar, and it is interesting to note that also from the phytochemical analysis it was found to be the richest of anthocyanins. These metabolites indeed can reach the brain *in vivo* and their metabolites are able to cross the blood-brain-barrier [71,72]. Similar considerations were made by Leong [73], who observed that cherry cultivars richest in anthocyanins were more effective in protecting Caco-2 cells from H_2O_2-induced damage compared to other cultivars. On the contrary, a study of cherry extracts found that those richest in anthocyanins had the least protective effect on the oxidative stress of cells, though not induced by hydrogen peroxide, but by tert-butyl-hydroperoxide [74]. To better clarify the intracellular antioxidant mechanisms of these extracts we evaluated their ability to modulate two antioxidant enzymes: GR and NQO1. GR enzyme is an important part of the cellular antioxidant defense as it maintains the intracellular

ratio of reduced/oxidized glutathione (GSH/GSSG) [75]. The important antioxidant activity of NQO1 is related to its catalytic mechanism: in fact, it catalyzes exclusively the two-electron reduction of quinones to their corresponding hydroquinones, thus avoiding the generation of highly reactive semiquinones and ROS [76].

Other authors demonstrated that anthocyanins are able to up-regulate GR and NQO1 [77], but to our knowledge, this is the first time that this kind of up-regulation has been observed after cherry extract treatment. In the brain, BDNF has a key role in the survival, maintenance and regeneration of specific neuronal populations (58). Depletion of this neurotrophic factor has been associated with different neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (59, 60) and strategies able to increase its levels are considered of great therapeutic interest. All the tested extracts significantly increased BDNF levels and this seems not to be strictly related to the anthocyanin content, as also cultivars with a low TAI values had a strong effect on BDNF expression. Williams et al. (61) observed that a blueberry extract increased BDNF levels in mouse hippocampus and ascribed this effect not only to anthocyanins but also to flavonols.

Conclusions

Sweet cherries are fruits rich in phenolic compounds (i.e., hydroxycinnamic acids, anthocyanins and flavonols) which have been recognized to play a clear role in the prevention of several diseases. In this study, levels of these bioactive compounds, as well as *in vitro* antioxidant capacity, have been investigated in seven new sweet cherry cultivars obtained through a natural breeding program carried out at the University of Bologna, and thus called "UNIBO" cultivars. In conclusion, some of the new sweet cherry cultivars obtained after a long-standing breeding program at the University of Bologna have been demonstrated for the first time to provide considerable added value, both in terms of bioactive compounds and neuroprotective activity *in vitro*, compared to other accessions. These characteristics make it possible to define these newly bred cultivars as a functional food. The variability observed in health-promoting phenolic compound content represents an excellent tool for selecting the best parents to be used in future breeding programs aimed at further improving the nutraceutical value of future sweet cherry cultivars, in order to obtain a real "superfood". Since oxidative stress is currently regarded as one of the most relevant conditions behind the development of many chronic-degenerative diseases, it becomes extremely important to consume an appropriate diet, rich in foods able to prevent the onset of such diseases.

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Malus domestica **Borkh.**

Ancient apple (*Malus domestica* Borkh.) cultivars as a source of chemopreventive substances: analysis of phytochemical profile and antioxidant capacity of peel and flesh

Unpublished data

Apple: general information and nutritional profile

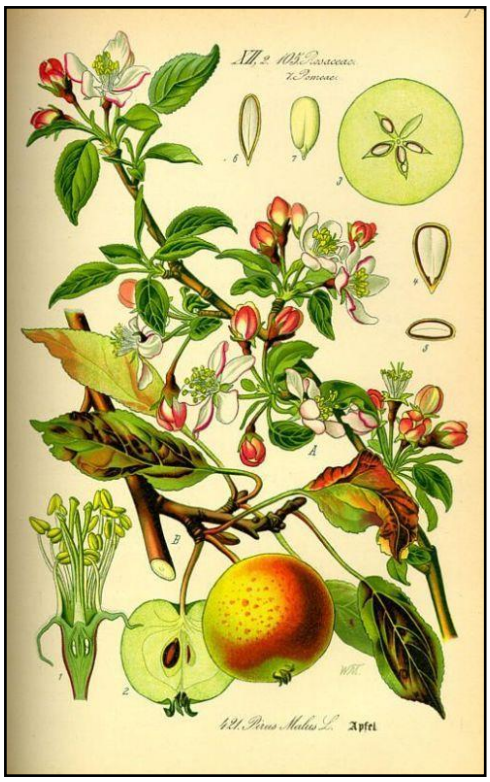

Figure 32: Malus domestica Borkh. *(syn. Pyrus malus* L.*)*

The domesticated apple, *Malus domestica* Borkh. (*Malus* x *domestica* Borkh.), belongs to Rosaceae family, subfamily Pomoideae and tribe Pyreae (Maleae) (Velasco et al., 2010; Donno et al., 2012), and is one of the most important fruit crops in temperate countries. The ancestor of the cultivated apple tree was Identified as the wild apple *Malus sieversii* (Ledeb.) M. Roem., which originated from mountains of central Asia, and later introduced into Europe and Egypt. Apple tree is a small/medium deciduous tree, with a single trunk and a broadly spreading canopy. Wild trees grow up to 10-15 meters height, while cultivated ones are shorter, reaching a maximum of 5 meters. The bark is brown-red, smooth, tomentose in the distal part, with well evident lenticels. Young stems and twigs are tomentose, while older ones are glabrous. Leaves are alternate, of an intense green color, of variable shape, from cordiform to obovate, of various dimensions as well, with serrated margins. The upper page is generally glabrous, more rarely tomentose, while the lower one is tomentose; the flap can be flat, concave or wavy. The petiole has different length, depending on varieties, and is provided with rather big stipules, deciduous. Flowers are usually terminal on spurs, borne in groups of 4-6, in inflorescences often described as corymbs which bloom starting from the central flower, for about 10-15 days in the month of April. The flower is a perfect flower, formed by five lobed calyxes, persistent in young fruits, while caducous in some species, at the ripening of the fruit or shortly before, and by five white-to-red petals of different shapes and sizes. Stamens are about 15-20 with yellow anthers, the ovary is infertile, divided into 5 lodges containing each two ovules and provided with 5 filiform styles with yellowish stigmas. The fruit of the apple tree is of ellipsoid to obovoid shape ,

more than 5 cm in diameter and weighing 200-350 grams; it is actually a false fruit, called **pome**, which derives from the growth of the floral receptacle and consists of an epicarp (with a cutinized and waxy epidermis), a fleshy mesocarp of white or cream white color, more rarely yellow or vinous red, and a leathery endocarp composed of 5 lodges, wrapped in 5 carpels, containing 2 smooth, shiny and dark brown seeds [1,2] (fig.32). Thanks to the domestication occurring over the centuries, we have the plant we know today [3]. This long-lasting process led to thousands of cultivars worldwide, most of which were then lost or abandoned; to date, In most developed countries, the market is dominated by only one hundred commercial varieties, the most famous of which are certainly "Fuji", "Delicious", "Golden Delicious", "Gala" "Granny Smith", "Idared", "Jonagold", "Braeburn", "Cripps Pink", "Jonathan", "Elstar" and "Mcintosh" [4]. Apples rank, especially in the western world cultures, number one or two (together with bananas) in terms of fruit consumption [5], thus contributing significantly to the "5-a day" recommendation, given by the WHO. A portion of one apple is approximately 200 grams and provides about 116 kcal (485 kJ), 79% of the total is edible portion. The following table (table 2) shows the nutritional values, both for macronutrients and for micronutrients.

Macronutrients (g)		**Micronutrients: mineral salts(mg)**		**Micronutrients: vitamins (mg)**	
Water	160.0	Sodium	2.2	Thiamine (B_1)	0.04
Carbohydrates	30.0	Potassium	239.0	Riboflavin (B_2)	0.06
Proteins	0.6	Iron	0.3	Niacin (B_3)	0.2
Lipids	0.4	Calcium	13.4	Vit A (ret. eq.)	19.0 (µg)
Fiber	5.4	Phosphorus	24.5	Ascorbic acid (vit. C)	10.3
				Alpha tocopherol (vit. E)	0.4
				Vit. K	2.1 (µg)

***Table 2**: nutritional profile of a medium-sized apple (about 200 grams). [6]*

Moreover, apples are generally low in proteins and lipids, and comparatively low in dietary fiber (consisting mainly of cellulose, hemicelluloses, lignins and pectins [7]), while they are particularly rich in sugars, in particular simple ones (glucose, sucrose and fructose), which contribute to almost all of the calorie intake.

The content In micronutrients, such as minerals and vitamins, is not very high, with the exception of potassium, nonetheless it constitutes a good integration of these elements [8]. An interesting aspect about eating apples is **peeling**: with the exception of organic crops, there has recently been a tendency to discard the peel of apples, because its waxy and lipophilic nature tends to favor the accumulation of harmful substances, such as pesticides [9]. This practice, however, causes both the loss of beneficial lipophilic compounds, such as triterpenes and phytosterols, as well as of certain polyphenols, whose concentration in the peel is 4-10 times higher than in the flesh. The red pigmentation of some apple varieties is due to the presence of anthocyanins, which are irretrievably lost during the peeling of the fruit, along with most of ascorbic acid (up to 40% resides is found in peel) and fiber (which amounts to 50%) [10]. The nutraceutical potential of the apple lies precisely in its phenolic compounds, which are mainly represented by hydroxycinnamic acids, flavan-3-ols and proanthocyanidins, anthocyanidins, flavonols, and dihydrochalcones. As for hydroxycinnamic acids, they are mainly represented by caffeoylquinic and *p*-coumaroylquinic acids and are more abundant in flesh compared to peel. On the contrary, flavonols and anthocyanins are mainly concentrated in the fruit peel [11]. The class of dihydrochalcones is represented mainly by phloretin glucoside (called phlorizin) and the xyloglucoside [11]. As concern flavan-3-ols, they can be found in both monomeric (catechin and epicatechin) and polymeric (procyanidins) forms, the latter being of B-type, i.e., flavan-3-ols are joined by C4-C6 or C4-C8 bonds. They are constituted of mainly (-)-epicatechin, which may be present as an extension unit or as a terminal unit; (+)-catechin is present exclusively as a terminal unit [11]. Flavonols are often in glycosylated forms, and predominant sugars are galactose, glucose, rhamnose, arabinose, and xylose. Distribution of secondary metabolites in apple is not uniform at the tissue level, and their concentration fluctuates in relation to many factors [12]. It depends on the cultivar, fruit maturity, growing conditions, leavening, harvesting, storage, and infections undergone [13]. Concentrations of total phenols are usually higher in apple skin than in pulp; however, concentrations of some individual compounds, for example, chlorogenic acid, catechins, and procyanidins may be higher in apple pulp than in apple skin, both in the early stages of development and before harvest. Concentrations of most flavonoids in apple skin were found to decrease with fruit development [12].

"*Old but gold*": rediscovering ancient apple cultivars

Apples are certainly among the most consumed fruits in the world and, from many years, the most popular cultivars on the market are Gala, Fuji, Golden Delicious, Pink Lady, which are the result of intensive selective breeding programs, developed over the years with the aim of obtaining fruits with an appreciable appearance and taste, as well as resistance to diseases, and increased shelf-life. Obviously, this process has led to a dramatic loss of biodiversity in apple cultivation [14]; if in the past there were more than 7500 varieties all over the world, to date most of them have been completely neglected and forgotten, due to the selection of a few high-yield and more profitable cultivars [15]. These breeding programs were driven by market demands, which request for apples to have specific quality traits: a certain level of sugars, organic acids and phenolic compounds which give each apple cultivar their characteristic taste. Indeed, breeding new cultivars is one means by which polyphenols may be altered in apple fruit. Conversely, ancient apple cultivars are characterized by unconventional quality traits, such as shape, peel color, nutritional value and organoleptic traits (such as crispness, juiciness and flavor), in addition they are richer in phenolic compounds, which are responsible for both color and taste of the fruit. Oligomeric proanthocyanidins contribute to bitterness, while the polymeric ones are responsible for astringency [16], thus making some cultivars not suitable as food [17]. **So why is it so important to rediscover these forgotten cultivars and give them value?** As well as having a higher nutritional value, preserving ancient genetic material of apples would contribute to safeguard crop biodiversity and the historical and cultural heritage they represent [18]. These ancient fruits indeed, have a very deep connection with the territory from which they come, and reflect particular agricultural traditions [19]. Moreover, autochthonous genetic heritage can be a source of genes that can be used to enhance the resilience of commercial varieties toward biotic and abiotic stress, or they can represent excellent progenitor candidates for future natural breeding projects [20]. Since ancient cultivars are more adapted to environmental changes, they usually require fewer treatments and field operations, in comparison with commercial cultivars. In Italy, until the mid-twentieth century, hundreds were the cultivars produced, however a few years later there was a decline of many of them, to favor highly specialized orchards, depleting the local germplasm [21]. Currently, more than 70% of orchards produce only one cultivar, **Golden Delicious**. In the last 10 years,

several research projects have been carried out in Northern Italy aimed at rediscovering and revaluing local fruit germplasms to conserve a resource directly related to the natural environment [22].

Ancient apple cultivars are still produced in Italy at level of local orchards, with over hundred varieties produced along the national territory, proving that the interest in conservation of the autochthonous genetic heritage of fruit species, is continuously growing. As a consequence, some neglected apple cultivars have been recently rediscovered, preserved and valorized [15,23]. "**Melannurca Campana**" an IGP apple, which has also been defined as "*the queen of apples*", is a clear example of a revalued variety, as it has been cultivated in the Campania region (Italy) for more than two millennia [24]. Several pharmacological effects have been demonstrated for this apple variety, including an antiproliferative action on MCF-7 breast cancer cells [25]. Also, Marche region has several ancient varieties, recently rediscovered and revaluated, thanks to their nutraceutical potential, such as "**mela Rosa dei monti Sibillini**" [26,27], which has been recently recognized as a slow food presidium. A recent study reported the protective effect of an hydroalcoholic extract of this apple variety towards severe kidney injuries in rats, an effect linked to the presence, in these fruits, of some classes of polyphenols, particularly flavonols and proanthocyanidins [28]. Another example, still in Marche region (Italy), concerns the discovery of a new red flesh apple with a pleasing taste; this fruit has been renamed as "**Mela del Pelingo**" and a procedure has been started to obtain the patent. The juice of this apple showed very promising *in vitro* antiproliferative activity on cancer cell lines, possibly correlated with its rich polyphenolic pattern. Pelingo apple juice in fact, has been shown to act as antiproliferative in human breast cancer cell lines (MCF-7 and MDA-MB-231), and this activity is mediated by the overexpression of proteins involved in the regulation of the cell cycle and autophagy [29].

Although flavonoids and polyphenols are well known as antioxidants, a possible pro-oxidant effect has been associated with a pro-apoptotic function of these compounds on various types of cancer cells, particularly breast and colorectal cancer [30,31]. Their ability to influence the cellular pathways involved in the survival, growth, and proliferation of cells both *in vitro* and *in vivo* has also been demonstrated. Several studies have suggested that flavonoids exert inhibitory effects on growth of various kinds of cancer cells, mediated by different molecular targets and acting through diverse metabolic pathways [32]. However, the precise mechanisms responsible for the anticancer effect of flavonoids are still not thoroughly clarified [33]. Flavonoids can bind to cell membranes, penetrate *in vitro* cultured cells, and modulate some metabolic activities [34]. Among the suggested activities for flavonoids, it is worth mentioning the mitigation of oxidative damage, the inactivation of carcinogen, growth Inhibition, promotion of differentiation, induction of cell cycle arrest and apoptosis, impairment of tumor angiogenesis, and suppression of metastasis [35,36]. The potential anti-tumor effect of flavonoids has never been explored in hepato-biliary and pancreatic tumors. Their incidence is increasing in Italy and the S. Orsola-Malpighi hospital in Bologna is one of the most important National centers in the care of patients suffering from these neoplasms. Accumulating evidence indicate that these types of cancer develop through the accumulation of genetic and epigenetic alterations, which are influenced by several factors, including the diet [37]. Based on the numerous evidences in favor of the nutraceutical and health-beneficial effects of apple fruits, and In the context of a reevaluation of ancient cultivars of this plant as a source of beneficial compounds, a project was developed with the aim of testing the anti-carcinogenic and anti-proliferative activities of extracts of ancient apple cultivars on tumor cell lines of cholangiocarcinoma, hepatocarcinoma and pancreatic cancer. This project comes from the collaboration among our research group, the one of Prof. Stefano Tartarini of the Department for Agricultural and Food Science, and the team of Prof. Giovanni Brandi of the Department of Experimental, Diagnostic and Specialty Medicine of Bologna University. The research carried out during my PhD represented the first part of this project and concerned the investigation of the chemical profile of phenolic compounds and antioxidant capacity of 35 ancient cultivars of apples of various Italian origins,

compared with that of two widely consumed reference cultivars (**Golden Delicious** and **Fuji**). The objective of this first part of the project was to Investigate the differences in phytochemical profile among all ancient cultivars and ultimately to select the most different ones, which will then be utilized for biological assays. This analysis was carried out on separate flesh and peel, since it is known that the latter possesses a higher concentration of some phenolic compounds, compared to the flesh [13,38]. The compounds of interest were detected in peel and flesh both in free and soluble-conjugate form. Levels of bioactive compounds were determined in extracts by both spectrophotometric methods and HPLC-DAD analysis. The antioxidant capacity was also evaluated using different *in vitro* assays (ORAC and ABTS). The qualitative and quantitative differences in the phytochemical profile among all cultivars were used to select the most different ones in terms of their nutraceutical potential. The second part of the project will be carried out to define a possible antitumoral effect, both in terms of chemoprevention and integrative therapy, of extracts of the selected varieties. In particular, the following biological activities will be performed:

- the *in vitro* **chemopreventive** capacity of the extracts, on normal cholangiocytes and hepatocytes cell lines treated with carcinogens.
- the *in vitro* **antiproliferative** effect of the extracts alone or in combination with specific chemotherapeutics in cell lines of biliary tract cancers.
- the antitumoral capacity, even in the presence of metastases, of the extracts alone or in combination with a specific chemotherapeutic *in vivo* on animal models derived from patients with cholangiocarcinoma.

If the results will be promising, these fruits could be used as chemo-preventive or supplementary therapeutic agents in both healthy people and oncology patients. The antiproliferative activity of apple polyphenols extracts on *in vitro* and *in vivo* models, could bring to light their potential as a new strategy for integrated therapy, to be combined with standard therapies, entering fully into the nutritional changes required primarily by patients. This study paves the way for the development of foods that are palatable and functional to human health, specifically to that of patients. The discovery of such potential in these ancient apple varieties, could therefore contribute to their full recovery and valorization, with a positive impact for the territories in which they are cultivated.

Plant material and sampling procedure

Apple plants belonging to different genotypes were grown under conventional agronomic practices in specialized orchards, located in the Experimental Teaching Center of the Department of Agricultural and Food Sciences of the University of Bologna, located in Cadriano, Granarolo dell'Emilia (Bologna). The study plan included fruits of two commercial cultivars, **Fuji** and **Golden Delicious, and of** thirty-five ancient cultivars of different origin, which are part of the germplasm collection of the Department. The list of cultivars utilized for the study is shown in table 3. The abbreviated names indicated in brackets will be used from here on. For each cultivar, three apples fruits were collected from three different trees, at full maturity, in harvest season 2018.

Belfiore di Trento (PD)	Renetta grigia di Torriana (RGT)	Arkansas black (AB)	Bella d'barge (BDB)
Idice 2 (ID2)	Bella del giardino (BDG)	Mela del Giappone (MDG)	Campanino (CA)
Idice 4 (ID4)	Muso di bue (MBD)	Mela di montagna (MDM)	Mela bastone (MB)
Calvilla san salvatore (CSS)	Scodellino (SCO)	Commercio (CFO)	Abbondanza (ABB)
Pum tosc (PT)	Calvilla bianca d'inverno (CBI)	Mela zitella (MZ)	Abbondanza rossa (ABR)
Rus canaviot (RC)	Gambafina (GF)	Mela rosa romana (MRR)	Capo d'asino 1 (CDA)
Mela rozza (MRZ)	Garola (GA)	Mela tinella (MT)	Democrat (DEM)
Rosa (RFi)	Gris Canaviot (GC)	Durello di Forlì (DDF)	Spitzlederer (SP)
111 (UNIBO)	005 (UNIBO)	035 (UNIBO)	

Table 3: Name of the apple cultivars studied, with abbreviations in brackets.

Reagents and standards

Methanol, acetic acid, diethyleter, ethyl acetate, sodium hydroxide, Folin-Ciocalteau phenol reagent, gallic acid, sodium carbonate, sodium nitrite, aluminum chloride, 6-hydroxyl-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox®), fluorescein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), monobasic and dibasic sodium

phosphate and solvents used for chromatographic purposes (water; *ortho*-phosphoric acid 85%; formic acid, acetonitrile, all of HPLC grade) were purchased at Sigma Aldrich (Milano, Italy) and filtered via 0.45-µm polyamid filters (Ahlstrom-Munksjö, Helsinki, Finland).

Pure standards (>99.5% purity in powder form) of phenolic acids and flavonoids (chlorogenic acid CAS: 327-97-9, caffeic acid CAS: 331-39-5, phlorizin CAS: 60-81-1, quercetin-3-O-galactoside CAS: 482-36-0, quercetin-3-O-rhamnoside CAS: 522-12-3, quercetin-3-O-glucopyranoside CAS: 482-35-9, (+)-catechin CAS: 154-23-4, (-)-epicatechin CAS: 490-46-0, were purchased from Extrasynthese (Genay, France). All standards were prepared as stock solutions at 1 mg/mL in methanol and stored in the dark at -20°C for less than three months.

Extraction procedures

Free phenolic fractions

Flesh and peel were separated in each fruit, immediately frozen in liquid nitrogen and freeze-dried. Samples of flesh and peels from fruits collected from the same tree were mixed and finely ground in a knife mill (IKA A11, Staufen Germany) for 4 × 15 s periods. The fine powder was then subjected to the "coning and quartering" sampling procedure and three biological replicates were carried out (fig. 33).

Figure 33: freeze-dried sample of apple flesh (on the left), subsequently subjected to milling (on the right), prior to sampling.

Phenolic compounds were extracted from samples following the protocol by Mattila et al., [39] with modification. A 0.5-g aliquot of freeze-dried powder was extracted twice with 5 mL of a mixture of methanol/acetic acid 10% (85:15; V/V) and homogenized (IKA Ultra Turrax T-18 Staufen Germany) for 2 min at speed 5.5.

The suspension was then sonicated, with an ultrasonic bath (50/60 Hz and 500 W), for 30 min at 35°C. After centrifugation (CL10 Thermo Fisher Scientific Carlsbad, CA, USA) at 4°C for 5 min at 1400×g, the supernatant was transferred into another tube and the pellet was subjected to the second extraction with the same procedure. The supernatants were merged, mixed and filtered through Grade 44 (3 μm) ashless filter paper. The final volume of limpid liquid obtained was recorded.

The extract was filtered again through a syringe filter (nylon, 0.22 μm pore diameter, from Thermo Fisher Scientific). The supernatant was used to analyze both the free and the soluble-conjugated forms of phenolics.

Soluble-conjugated phenolic fractions

An 8-10 mL aliquot of the supernatant has been added to 12 mL of distilled water and 5 mL of 10 M NaOH, in order to carry out an alkaline hydrolysis. The solution was thoroughly mixed, and bubbled under nitrogen flow for 1 minute, to prevent any oxidative processes. Alkaline hydrolysis was conducted at room temperature for 16 hours under stirring, after that, the solution was acidified until pH 2 with concentrated hydrochloric acid before proceeding with three consecutive extractions of phenolic compounds with a mixture of diethyl ether and ethyl acetate (1:1; V/V). The merged organic phases were evaporated, and the dry residue was then resuspended in 1,5 mL methanol.

Spectrophotometric determination of total polyphenol and total flavonoid content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu assay on apple peel and flesh extracts using a Jasco V-630 double beam spectrophotometer, as described by Singleton & Rossi (1965) with some modifications [40,41]. For the setup of calibration curves, 440 μL of 0.2 N Folin–Ciocalteu phenol reagent in water and 100 μL of aqueous gallic acid solution at different concentrations (0-100 μg/mL), or 100 μL of distilled water (blank solution), were mixed in a 2-mL polypropylene vial and allowed to react for 10 minutes, and then 440 μL of 7.5% sodium carbonate (w/v) aqueous solution was added. For sample analysis, the standard solution was replaced with 100 μL of suitably diluted sample. The vial was then incubated in the dark at room temperature for 2 h, then the

absorbance of the solution was read at 765 nm. Results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

The total flavonoids content (TFC) was measured according to Tang et al. (2015) [41], with modifications. For the setup of calibration curves, 440 µL of aqueous 0,066 M $NaNO_2$ and 100 µL of aqueous catechin solution at different concentrations (7.8-250 µg/mL), or 100 µL of distilled water (blank solution), were mixed in a 2-mL polypropylene vial and allowed to react for 5 minutes, then 60 µL of aqueous 0,75 M $AlCl_3$ solution was added and waited for another 5 minutes, lastly 400 µL of 0,5 M NaOH are added. For sample analysis, the standard solution was replaced with 100 µL of suitably diluted sample. Results were expressed as milligrams of catechin equivalents per gram of dry weight (mg CAT/g DW).

HPLC-DAD Determination of phenolic compounds

The method for phenolic acids analysis was adapted from Mattila et al., [39]. Elution was carried out with a mixture of solvent A (50mM phosphoric acid) and solvent B (acetonitrile), with a composition gradient ranging from 97% to 50% of solvent A and flowing at 0.7 mL/min. Signals at 287 were used for identification of phlorizin, while at 329 nm for chlorogenic and caffeic acids. The method for flavonoids analysis was adapted from Wojdylo et al., [42]. Elution was carried out with a mixture of solvent A (water/formic acid 95,5/4,5; V/V) and solvent B (acetonitrile), with a composition gradient ranging from 97% to 36% of solvent A and flowing at 0.5 mL/min. Signal at 280 were used for the identification catechin and epicatechin; while at 360 nm for quercetin-3-O-galactoside (or hyperoside). The extracts were injected into a Jasco (Tokyo, Japan) HPLC-DAD system, which consisted of a PU-4180 pump, an MD-4015 PDA detector and an AS-4050 autosampler. The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm × 3 mm I.D., 3.5 µm). Stock solution of standard were prepared in methanol at concentration of 1000 PPM, and calibration curves were built up for each standard compound in the concentration range between 1,5 and 100 PPM using HPLC solvent A as diluent. All calibration curves obtained showed a correlation coefficient greater than 0.9987. Injection volume was 50 µL for all determinations.

In vitro antioxidant activity assays

ORAC assay was carried out as previously described in Materials & Methods in section "*Prunus avium* L. - Characterization of New Sweet Cherry cultivars for their phenolic profile, antioxidant activity and neuroprotective potential." **ABTS** assay was carried out as described by Miller et al. (1993) with some modifications [43,44], using a Jasco V-630 double beam spectrophotometer. 990 µL of properly diluted 2 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 5mM PBS buffer (pH 7,4) and 10 µL of Trolox solution at different concentrations (0.05-1.00 mM), or 10 µL of PBS buffer (blank solution), or 10 µL of 1:2 diluted sample (in methanol), were read at 734 nm. Calibration curves were set up plotting the discoloration ratio as a function of Trolox concentration. The antioxidant capacity of the sample, expressed as Trolox equivalents (TE), was calculated interpolating on the calibration curve.

The data obtained are expressed as mean ± standard deviation (SD), which was calculated on the three biological replicates of the samples. Multivariate statistical analysis was conducted similarly to what has already been described at in section "*Prunus avium* L. - Characterization of New Sweet Cherry cultivars for their phenolic profile, antioxidant activity and neuroprotective potential."

Total polyphenol and flavonoid content in peel and flesh of apple cultivars

TPC provided preliminary information on both amounts of phenolic compounds in extracts and their tissue localization (flesh or peel). In the graph reported in figure 34 the TPCs in all analyzed cultivars in **flesh** extracts is shown; their concentration in the two

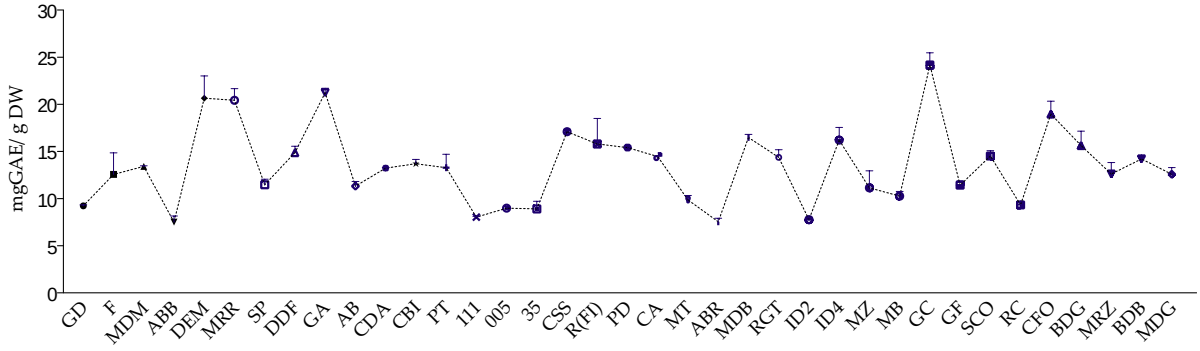

Figure 34: TPC of apple flesh extracts, expressed as mg GAE/g DW. Data are the mean ± SD of three biological replicates.

reference cultivars (F and GD) was rather similar, ranging between 9.2 and 12.5 mg GAE/g DW. Most ancient cultivars entered within this range of values, while some of them turned to have higher concentrations than reference cultivars, and these were DEM, MRR, GA, CSS and GC, the latter reaching 24.2 mg GAE/g DW.

Apple **peel** extracts showed a quite similar pattern (fig. 35) to that found in flesh. Reference apples varied between 12.5 and 14.1 mg GAE/g DW, and in the same range were also placed some of the ancient cultivars. Those with a higher content of polyphenols, compared to reference cultivars, were MDM, DEM, MRR, CFO which reached a level double than Fuji. Once again, GC was confirmed as the richest in polyphenols, with a level of 28.95 mg GAE/g DW.

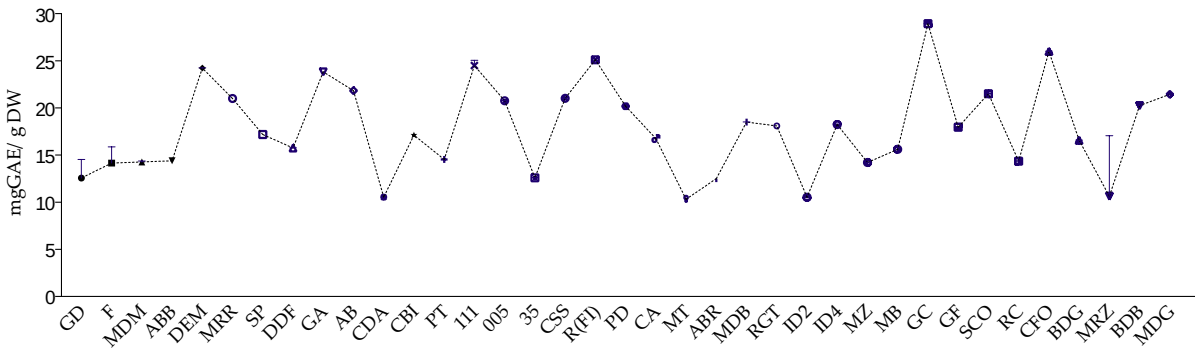

Figure 35: TPC of apple peel extracts, expressed as mg GAE/g DW. Data are the mean ± SD of three biological replicates.

The TPC found in peel of our ancient apple extracts are quite in line with data in literature, even though the comparison with data reported by other authors is not easy, since several parameters were demonstrated to have a great impact on the extraction efficiency of these metabolites, such as sampling, starting material (fresh *vs* freeze-dried), solvent-to-solid ratio used for extraction, duration of extraction, and sonication treatment applied, among others [45]. Moreover, some authors express data on FW basis, and the conversion to DW one can vary, depending on the relative water content. Results in the same order of magnitude were obtained by Raudone et al. (2017) on ancient Lithuanian cultivars [46], while Nkuimi Wandjou et al. (2020) reported higher total polyphenol content in peel samples of an ancient variety of pink apple (mela Rosa dei Monti Sibillini), in the range 36.5 to 55.7 mg GAE/g DW [26]. As regards TPC in the flesh, our results are consistent, or slightly higher, to those reported by other studies. For example, Preti and Tarola (2020) [23], found a slightly lower range of 4.50-8.75 mg GAE/g DW in flesh of ancient Friulian apples, as calculated by converting their data to DW basis, similarly to Berni et al. (2019) in ancient Tuscanian apples [47]. Although whole fruits were analyzed by these authors, without separating flesh from peel, levels of over 40 mg GAE/g DW were detected in some cultivars. It is worth to note that, in both studies, reference cultivars had the lowest levels of polyphenols among all cultivars, the difference being up to ten times, thus reflecting the same trend identified in our study.

The results of the total flavonoid content in apple **flesh** extracts are shown in figure 36. The reference cultivars had a similar value, about 1-1.5 mg CAT/g DW and most ancient apples fell within this range of values. Some ancient varieties had a concentration about 2 to 4-fold higher than reference ones, in particular DEM, MRR, SCO and BDB. GC was again the richest cultivar, reaching the highest concentration among all analyzed cultivars (4.74 mg CAT/g DW).

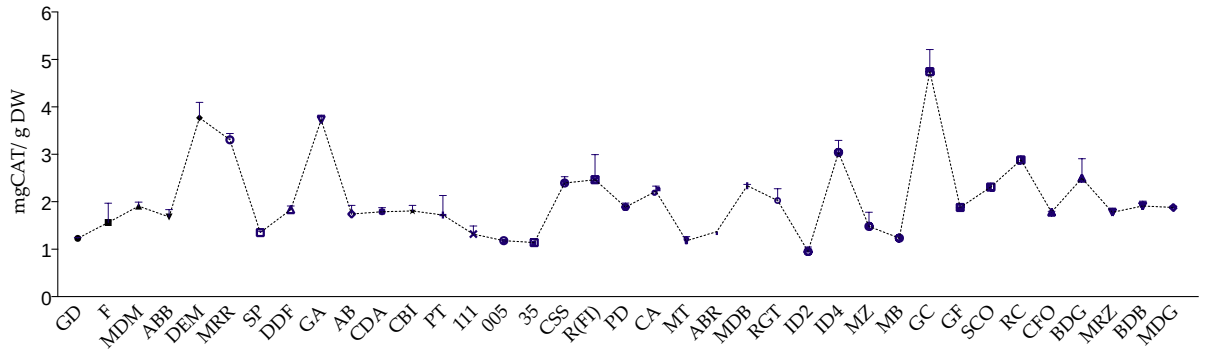

Figure 36: TFC of apple flesh extracts, expressed as mg of catechin (CAT) equivalents/g of dry weight. Data are the mean ± SD of three biological replicates.

The pattern of total flavonoids within apple **peel** extracts was much more variable than that of the flesh (fig. 37). The reference apples had levels below 1 mg CAT/g DW. The richest ancient cultivars were DDF, GA, ABR, ID4, SCO and MDG, the latter having a concentration of 3.64 mg CAT/g DW.

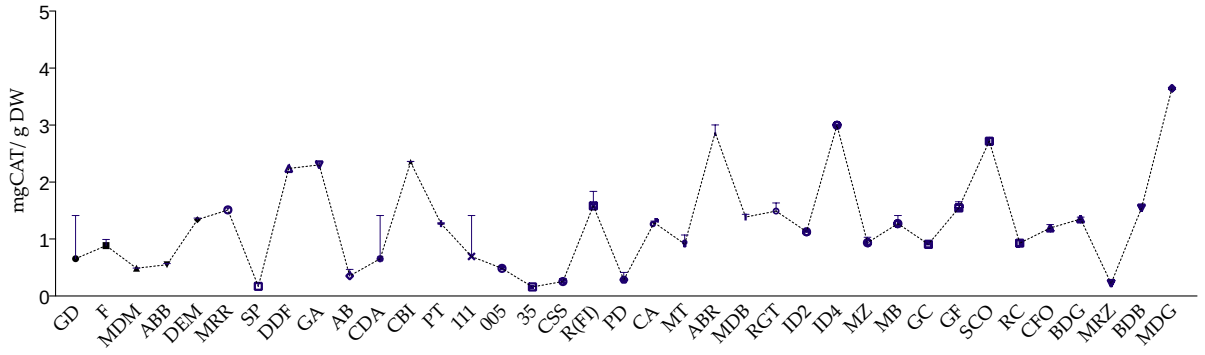

Figure 37: TFC of apple peel extracts, expressed as mg of catechin equivalents/g of dry weight. Data are the mean ± SD of three biological replicates.

Generally, the concentration of flavonoids is higher in the peel than in the flesh [48,49], whereas, based on our preliminary analysis carried out through spectrophotometric assays, the difference In the concentrations between the two parts of the fruit was not so pronounced. Nevertheless, it should be considered that these spectrophotometric methods only provide a general overview of polyphenolic and flavonoid content, since they are not so specific, and thus they should always be integrated with chromatographic analysis to get a more realistic overview [50].

Individual phenolic compound profile

Phenolic acids in apple extracts

Determination of phenolic acid content in apple samples began with chromatographic analysis of pure standards, as reported in Material and methods section. Phlorizin, although being a dihydrochalcone, was analyzed with the same method used for identifying phenolic acids and has the maximum absorption peak at 287 nm. The chromatographic profile of the mixture of phenolic acid standards and phlorizin is shown in figure 38, while overlaps between the standard mixture, a representative ancient cultivar (GA), and a reference cultivar (F) are shown in figure 39 (A and B), with the aim of highlighting the peak of chlorogenic and caffeic acid, respectively.

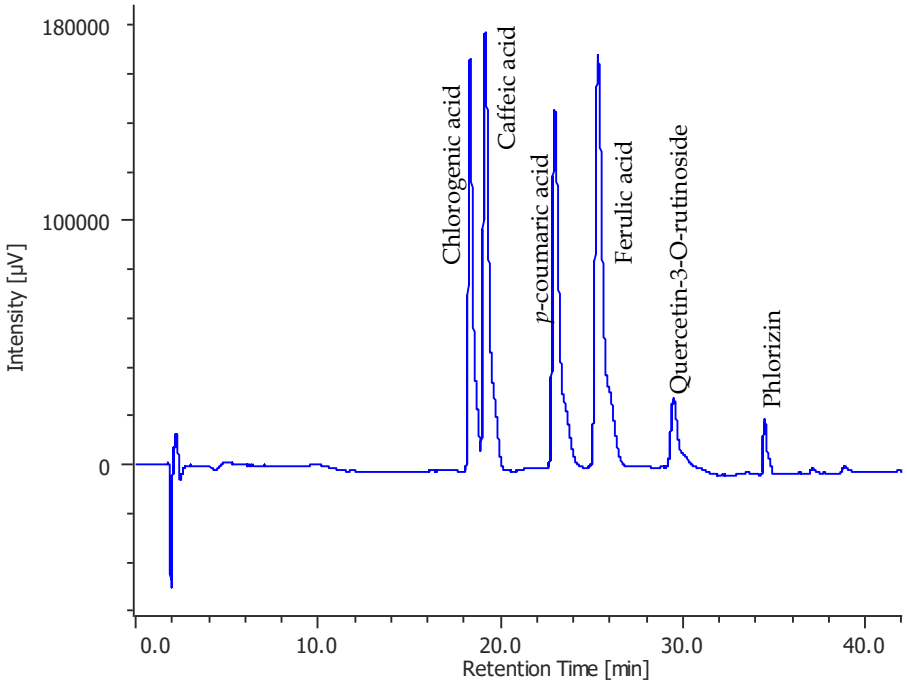

Figure 38: Chromatographic profile of the standard mix at 15 PPM concentration, 329 nm wavelength.

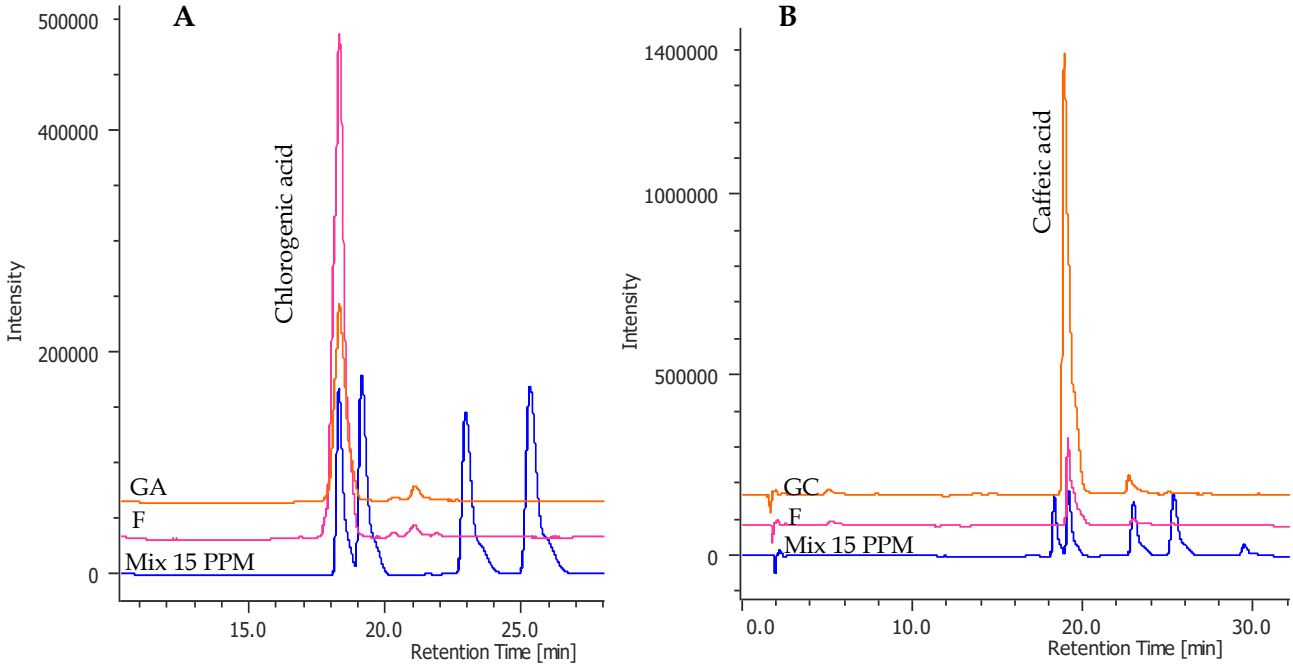

*Figure 39: Overlay of chromatographic profiles (wavelength 329 nm) of the standard mix and **free flesh extracts** (1:2 dilution) (**A**) and **hydrolyzed flesh extracts** (1:5 dilution) (**B**).*

The major phenolic acids in apple fruits were two hydroxycinnamic acids, namely chlorogenic and caffeic acids. Chlorogenic acid was found in the flesh of all analyzed apple cultivars, and its levels ranged from 1.0 to 21.0 mg/g DW (fig. 40), thus showing great variations depending on genotype.

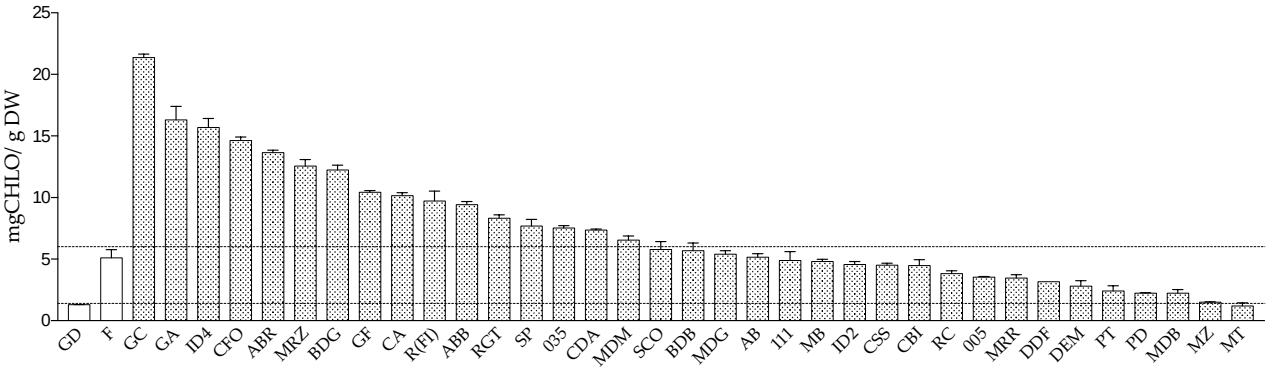

Figure 40: Chlorogenic acid levels of apple flesh extracts (mg/g DW). Data are the mean ± SD of three biological replicates.

Golden Delicious was the poorest cultivar in chlorogenic acid content, and a difference was found respect to Fuji, which showed a three-fold higher concentration. All ancient cultivars, except MZ and MT, were found to be richer in chlorogenic acid than GD, and 15 out of 35 samples had a chlorogenic acid concentration higher than Fuji. Among these, seven cultivars (i.e., GC, GA, ID4, ABR, CFO, ABR, MRZ, BDG) were particularly rich in chlorogenic acid, with values ranging from 12 to 21 mg/g DW. The cultivar GC certainly stood out from all the other ones, with a concentration of this caffeic acid derivative four- to twenty-fold higher than Fuji and GD, respectively.

As regards peel samples, chlorogenic acid was found at lower levels compared to the flesh, ranging from 0.37 to 11.0 mg/g DW, and in some cultivars (DEM, DDF, MT, MDB,

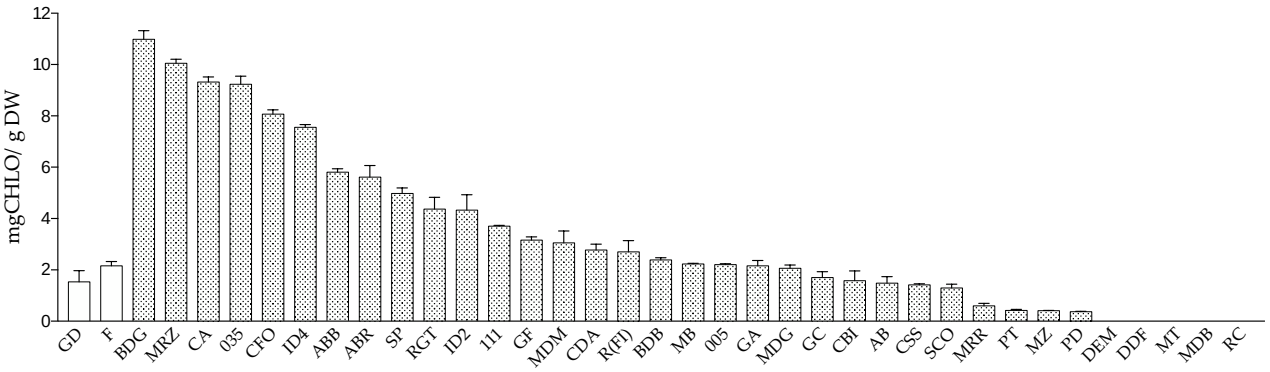

Figure 41: Chlorogenic acid levels of apple peel extracts (mg/g DW). Data are the mean ± SD of three biological replicates.

and RC), its levels turned below the detection limit. Commercial varieties recorded a value between 1.50 and 2.10 mg/g DW, and among the ancient cultivar, the richest in this caffeic acid derivative were BDG, MRZ, CA, 035, CFO, and ID4. Conversely, MRR, PT, MZ, and PD were the poorest varieties (fig. 41).

In general, higher amounts of phenolic acids were found in apple flesh compared to the peel [42] and chlorogenic acid is certainly one of the major compounds of this class in apple flesh, as reported in literature [51,52]. On average, our data on chlorogenic acid content in commercial and in some ancient cultivars were in line with results reported in literature [53]. In the study conducted by Belviso et al. (2013) [51] on the polyphenol composition of Italian ancient apple cultivars in three consecutive years, chlorogenic acid was reported to range between 0.13 and 2.1 mg/g DW in seven different genotypes, with fluctuations depending on the harvest year. Comparable results were provided by Iacopini (2010) [54] in four Tuscan ancient varieties, with values ranging from 0.12 to 3.15 mg/g DW, as well as by Nkuimi Wandjou et al. [26] in lyophilized pulp samples of Mela Rosa dei Monti Sibillini, which showed a concentration of chlorogenic acid in the range 0.17-1.95 mg/g DW, depending on areas of cultivation. Nevertheless, it is worth emphasizing the richness in chlorogenic acid of some of the ancient cultivars analyzed in our study, such as Gris Canaviot, Garola, Idice 4, and Scodellino, reaching in some cases more than 20-fold higher levels compared to the reference ones.

Chlorogenic acid is among the most distributed phenolic acids in plants, especially because it plays a defensive role towards pathogens [55], and several studies of basic research, as well as clinical trials, have demonstrated an inverse correlation between 5-caffeoylquinic acid consumption and a lower risk of metabolic syndromes and chronic diseases (44). Among the richest dietary sources of chlorogenic acid there are coffee seeds, potatoes, eggplants, artichokes and sunflower seeds, while among fruits, apples, pears and blueberries represent good sources as well [56]. Coffee seeds, of course, contain the highest concentration, equal to 6-12% w/w; on average, a cup of coffee contains 27 to 121 mg of chlorogenic acid, which can reach up to 300 mg depending on the variety [57]. Since some apple cultivars analyzed here showed a concentration of chlorogenic acid of 20 mg/g DW, which corresponds to about 4.0 mg/g FW, the consumption of these fruits would provide a contribution comparable to that of two-three cups of coffee.

Sut (2019) also found that some ancient Friulian apple cultivars being studied could be a valid alternative to green coffee, as they provided a good amount of chlorogenic acid [48]. It has been proven that the *in vivo* administration of green coffee bean extracts to mice decreased weight gain and accumulation of visceral fat, and this activity was due to chlorogenic acid and its related compounds, that seems to be involved in upregulating liver lipid metabolism [58]. Many studies have demonstrated other health-promoting effects of chlorogenic acid, and given its antioxidant capacity, it has been classified as a preventing agent in diseases related to cellular oxidative imbalance.

These biological activities include, among others, the anti-inflammatory, anticancer, antiepileptic, antidiabetic, and antihypertensive, as reviewed by Lara *et al*. 2020 [59]. Results of another *in vivo* study, demonstrated neuroprotective role of chlorogenic acid, against neuronal cells exposed to arsenite poisoning, through a strengthening of endogenous antioxidant machinery and by the up-regulation of pro-apoptotic genes in a dose-dependent manner [60]. There is a correlation between the exposure to different toxic metals and the development of neurodegenerative diseases, and therefore extracts of apple enriched in chlorogenic acid could represent an useful tool to alleviate this toxicity, and preventing other damages [61]. Caffeic acid, as well as its derivatives, exerts different health-promoting properties: antioxidant, anti-inflammatory and antineoplastic. Recent studies both *in vitro* and *in vivo* have demonstrated its potential against a very aggressive and high incidence type of cancer, hepatocarcinoma. This activity is carried out through several pathways such as prevention of ROS formation, pro-oxidant action, anti-angiogenesis and suppression of certain matrix metallopeptidases, as reviewed by Espíndola *et al*. (2019) [62].

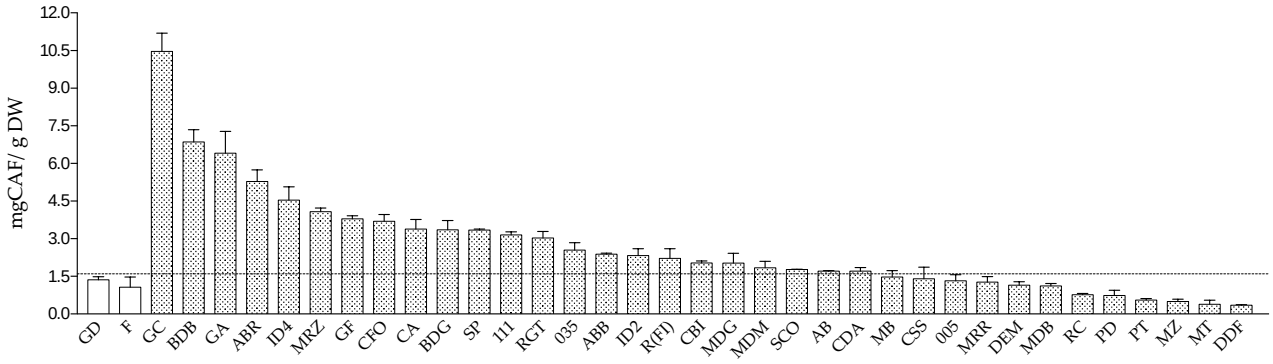

Figure 42: Caffeic acid levels of apple hydrolyzed flesh extracts (mg/g DW). Data are the mean ± SD of three biological replicates.

As concern caffeic acid, it was found more abundant in flesh extracts subjected to alkaline hydrolysis, with a level ranging from 1.5 to about 10.5 mg/g DW as shown in fig. 42. The reference cultivars showed a comparable level of caffeic acid, around 1-1.5 mg/g DW. Based on these concentrations, in some ancient varieties caffeic acid was lower (1 mg/g DW), while the BDB, GA, ABR, ID4, MRZ, and CFO showed values between 4.5 and 7.5 mg/g DW. Once again Gris Canaviot had the highest concentration, reaching 10.46 mg/g DW. A good correspondence was found between the cultivars with the highest levels of caffeic acid and those richest in chlorogenic acid, except for BDB. Regarding caffeic acid in hydrolyzed peel extracts, the results show a concentration range from 0.35 to 3.17 mg/g DW. Commercial varieties recorded a value between 0.91 and 1.11 mg/g DW, the richest ancient varieties being GC, 035, BDG, MRZ, and ID4 while the poorest DEM, MZ, and RC (data not shown). Caffeic acid is mainly concentrated in apple flesh rather than in peel [63], although in some cases a similar concentration in both tissues has been recorded [64]. What is interesting to note is that the ancient cultivars were richer in phenolic acids, even caffeic acid, when compared to commercial varieties. These compounds are usually responsible for the astringent taste and to the enzymatic browning of fruits, characteristics that are not appreciated by consumers. As a result, an intense breeding was carried out to drastically reduce the level of phenolic acid content of new apple cultivars [64].

Dihydrochalcones and flavonoids in apple extracts

The major phenolic compounds in the peel are flavonoids, belonging on the three classes of dihydrochalcones, flavan-3-ols and flavonols.

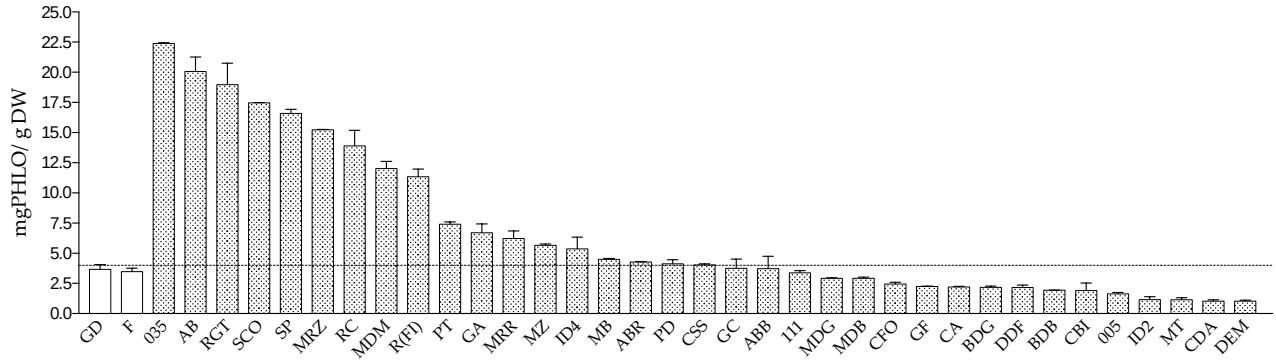

Figure 43: Phloridzin levels of apple peel extracts (mg/g DW). Data are the mean ± SD of three biological replicates.

The main representative compounds were phloridzin, catechin and epicatechin, and hyperoside, respectively. Fig. 43 shows the pattern of phlorizin in all the analyzed cultivars, which ranged from a minimum of <1 to a maximum of 22.5 mg/g DW, thus showing a variable content depending on genotype.

The two reference cultivars GD and F showed a similar content of this dihydrochalcone, which was about 4 mg/g DW. As concerns the ancient varieties, the highest concentration found were in 035, AB, RGT, SCO, SP, MRZ, RC, MDM, RFI, with values between 12.0 and 20.0 mg/g DW, while 15 out of 35 cultivars had phloridzin levels similar or lower than the two commercial varieties, and, among them, ID2, MT, CDA and DEM showed the lowest concentration. Figure 44 shows overlaid chromatograms with the peak corresponding to phloridzin in the standard mixture and in two peel extracts (GD and 035).

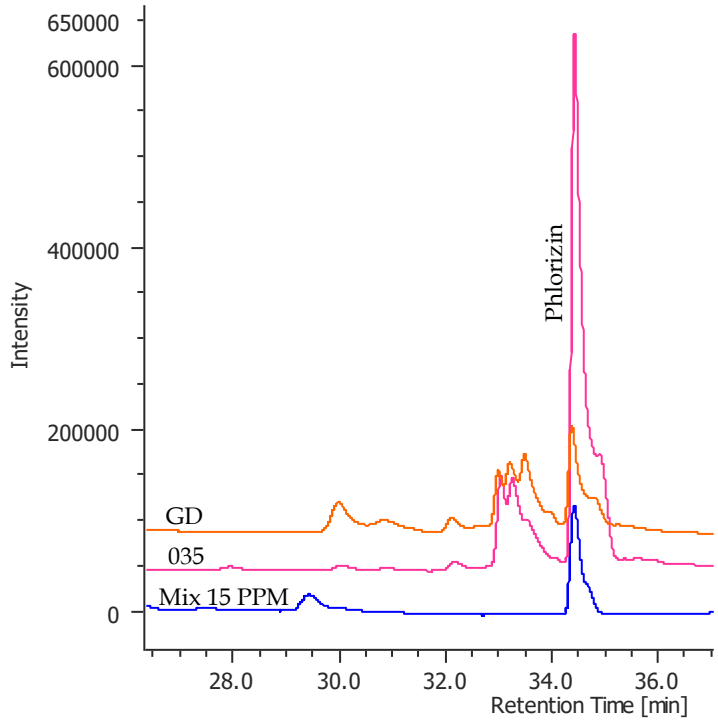

Figure 44: Overlay of chromatographic profiles (wavelength 287 nm) of the standard mix and free peel extracts (1:2 dilution) of 035 and GD.

As concern phloridzin concentration in apple flesh extracts, the results show a concentration range from 0.17 to 1.18 mg/g DW. Commercial varieties recorded a value between 0.21 and 1.18 mg/g DW, the richest ancient varieties being PT, GC, SCO and BDG, while the poorest MZ, BDB and MRZ. It is important to highlight that this compound was detected only in 21 of the 35 ancient varieties analyzed (data not shown).

Dihydrochalcones, such as phlorizin and its aglycone phloretin, are typical flavonoids of apple fruit, and can indeed be considered as a chemotaxonomic marker for the identification of apple cultivars [65]. Their main localization is the fruit peel, even though low amounts can also be detected in the flesh, as well as in the bark of the tree [66]. The richness of the ancient cultivars in dihydrochalcones has already been reported [48,67]. Our results are comparable, at least for some cultivar, with those found by Simonato et al. [67], who reported a phloridzin content ranging from 0.8 to 5.7 mg/g DW In five ancient Italian varieties, but In other cultivars here studied higher concentrations (up to 5-fold) were found.

Phloridzin is certainly one of the apple polyphenols that has most attracted the interest of the scientific community, since several studies have demonstrated Its efficacy in influencing glucose metabolism in humans, acting as a specific and competitive inhibitor of the Na/glucose cotransporters SGLT1 and SGLT2. While the latter is located only in the kidney, SGLT1 is located primarily in the small intestine as well as in the kidney.

By blocking these transporters and reducing sugar uptake from the intestine, while promoting at the same time the glucose excretion in the urine, phloridzin may significantly reduce the sugar load of the body. This could have a beneficial effect on patients who suffer of conditions of "pre" diabetes and of diabetes mellitus of type 2 [66]. A randomized *in vivo* study on healthy adults, male and female, from 18 to 70 years old, showed that the administration of apple extract enriched in phlorizin, was able to delay the post prandial glycemic peak, and consequently the absorption of sugars, through the gluco-regulation mechanism involving the carriers above mentioned [68]. A daily intake of phlorizin would have led to a significant decrease in total plasma cholesterol and triacylglycerols, favoring instead the accumulation of high-density cholesterol, through an increase in sterols excretion, a decrease in dietary cholesterol absorption and a parallel reduction in endogenous cholesterol synthesis *ex novo* [69]. This dihydrochalcone, like other compounds belonging to the class of polyphenols, have strong antioxidant properties that could be used to prevent diseases for which an oxidative stress has been identified as a key risk factor. Their role as reducing agents has shown a reduction in advanced end-product glycation, (AGEs) which are correlated with inflammatory bowel diseases [65].

Some of the varieties analyzed by us contain a surprisingly high content of phloridzin, especially in their peel, whose intake might contribute to the prevention of type 2 diabetes and to maintain low levels of intestinal inflammation. Thus, it seems reasonable to refer to them as real **superfoods**. Moreover, phlorizin and aglycon phloretin have been related to a resistance of fruit trees toward pathogens. These compounds in fact are hydrolyzed *in vivo* by various fungi to fluoroglucinol, phloretic acid and *p*-hydroxybenzoic acid, which are endowed with antibacterial and antifungal action [70], and the use of these compounds, from an agronomic point of view, could contribute to a greater sustainability in production [23].

Catechin was found to be the major flavan-3-ol monomer in free apple flesh extracts, with a value ranging from about 0.5 to 6.0 mg/g DW. In the two reference cultivars, the amount of catechin was rather similar, and did not exceed 1 mg/g DW. From the graph shown in figure 45 it is shown that some of ancient varieties of apple i.e., GC, ABB, GA, CA, were the ones with a concentration of 4 to 6-fold higher than references. It is interesting to note that the cultivar GC was still the richest, reaching 6.08 mg/g DW. As concern catechin concentration in apple peel extracts, the results show a concentration range from 0.10 to 3.55 mg/g DW. Commercial varieties recorded a value between 0.67 and 1.08 mg/g DW, the richest ancient varieties being CFO, MRZ, 035, CA and GC, while the poorest MDB, MZ and ID4.

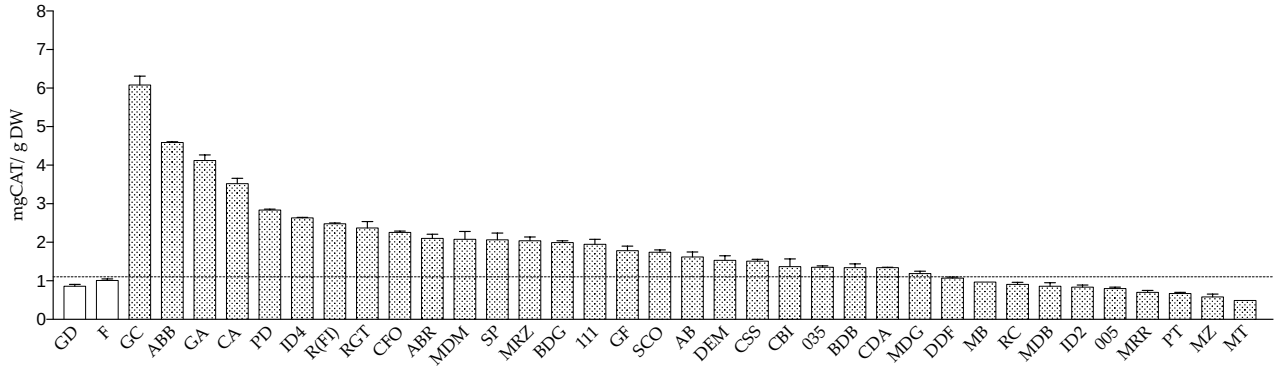

Figure 45: Catechin levels in apple flesh extracts (mg/g DW). Data are the mean ± SD of three biological replicates.

In the peel extract of DEM cultivar, this compound was not detected (data not shown).

In apple peel extracts, certainly the most abundant compound of the class of flavan-3-ols was epicatechin, whose concentration varied from 1.0 to more than 7.0 mg/g DW, as reported in fig. 46.

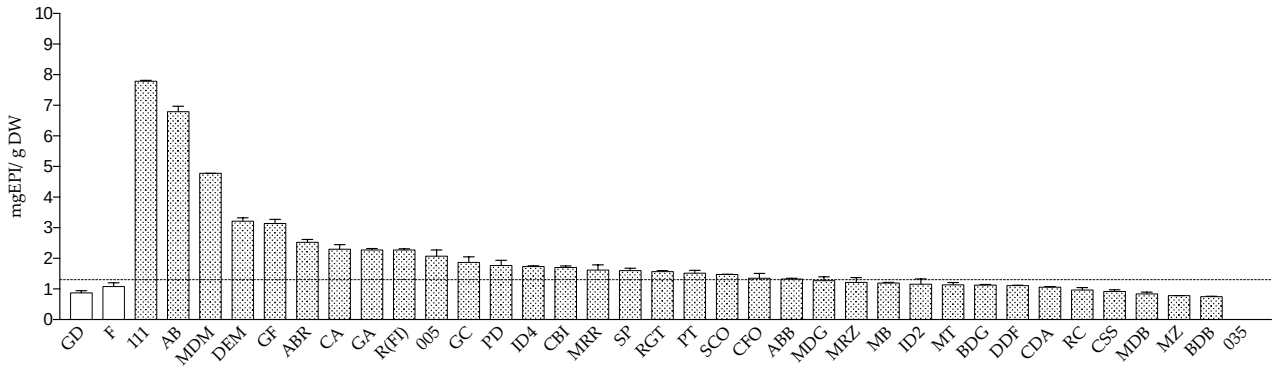

Figure 46: Epicatechin levels in apple peel extracts (mg/g DW). Data are the mean ± SD of three biological replicates.

Only in one cultivar (035), it was not detected. Again, the reference cultivars had a similar concentration of epicatechin, just above the milligram for gram DW. Even though the majority of ancient cultivars were in this range, five of them (111, AB, MDM, DEM and GF) showed a much higher level of epicatechin, with 111 having a concentration more than 7-time higher than commercial cultivars.

As for epicatechin amount in apple flesh extracts, the results show a concentration range from 0.08 to 1.98 mg/g DW, much lower compared to the peel. Commercial varieties recorded a value between 0.35 and 0.41 mg/g DW, the richest ancient varieties being DEM, MRR, MDB, GA and GC, while the poorest 005, ID2 and CFO (data not shown). It has been suggested that (-) epicatechin is more bitter than its stereoisomer (+) catechin [71]. Thus, it is probable that the cultivars Democrat, Mela Rosa Romana, Muso di Bue, Gris Canaviot and Garola turned particularly bitter if compared to the other varieties.

Our results about catechin and epicatechin showed that the former was more abundant in the flesh, while the latter in the peel. Although reports in literature show that these two compounds are more often found in the peel [21,72], no marked differences in the tissue localization of catechin have also been observed by some authors [21]. For example, Nkuimi Wandjou et al. [26] found similar concentrations of catechin in lyophilized pulp and peel extracts from the pink apple "Mela Rosa dei Monti Sibillini" (0.23 and 0.34 mg/kg, respectively), while epicatechin was more concentrated in peel compared to flesh (2.99 and 0.91 mg/kg, respectively).

According to their results, this Italian ancient cultivar turned to have concentrations of flavan-3-ols twice as high compared to the two commercial cultivars used as reference (Golden Delicious and Granny Smith). The values found in our reference cultivars (Golden Delicious and Fuji) are in line with those found in literature. Vrhovsek (2018), for example, reported less than 1.0 mg/g DW for both catechin and epicatechin in whole apples [73]. Catechin and epicatechin are the characteristic flavan-3-ols of unfermented green tea, but there are also found in black tea, coffee, berries, grapes and wine. Flavan-3-ols have been shown to act as antioxidants through several mechanisms including free radical scavenging, chelation of transition metals, as well as mediation and/or enzymatic inhibition [74]. Furthermore, the cell-death-inducing effect of catechins has been confirmed in several *in vitro* cancer models: H1299 human non-small cell lung cancer, MCF-7 breast cancer, stomach cancer, liver cancer and DU-145 prostate cancer [75].
As concerns flavonols, table 4 shows their concentrations in free apple peel extracts. Hyperoside was found to be the major compound of this class, followed by quercitrin and isoquercetin. Regarding the former, it was found in a concentration range of 0.06 to 3.5 mg/g DW. In detail, commercial cultivars showed a level between 0.40 and 1.00 mg/g DW, while among the ancient apples, CFO surely stands out, followed by AB and 005. The poorest cultivars were 035, RGT, MRZ and SP. Quercitrin, on the other hand, was found to range from 0.11 to 1.77 mg/g DW. Commercial varieties ranged from 0.29 to 0.73 mg/g DW, while among the ancient CFO, 111, SCO, and AB had the best results, as opposed to PT, RGT, SP, and MRZ.

Finally, the concentration of isoquercetin was in the range of 0.06 to 1.03 mg/g DW. Golden Delicious and Fuji had levels of 0.12 and 0.24, respectively, while the richest among the ancient cultivars were DDF, MDM, SP, and MRZ, and the poorest were DEM, ID2, RC, and lastly was not detected in RGT.

Cultivars	Hyperoside Mean ± DS	Quercitrin Mean ± DS	Isoquercetin Mean ± DS
GD	0.32 ± 0.01	0.29 ± 0.02	0.12 ± 0.00
F	1.07 ± 0.10	0.73 ± 0.1	0.24 ± 0.01
MDM	0.57 ± 0.04	0.47 ± 0.02	0.19 ± 0.02
ABB	0.59 ± 0.02	0.44 ± 0.06	0.18 ± 0.01
DEM	0.61 ± 0.14	0.57 ± 0.09	0.25 ± 0.06
MRR	0.44 ± 0.00	0.41 ± 0.00	0.24 ± 0.00
SP	0.07 ± 0.00	0.11 ± 0.01	0.06 ± 0.00
DDF	0.23 ± 0.00	0.16 ± 0.00	0.12 ± 0.00
GA	0.87 ± 0.09	0.87 ± 0.06	0.36 ± 0.01
AB	**2.07 ± 0.03**	**1.1 ± 0.04**	0.53 ± 0.00
CDA	0.27 ± 0.00	0.19 ± 0.01	0.19 ± 0.01
CBI	0.35 ± 0.04	0.51 ± 0.11	0.21 ± 0.00
PT	0.21 ± 0.02	0.12 ± 0.01	0.15 ± 0.01
111	1.11 ± 0.07	**1.66 ± 0.1**	0.26 ± 0.00
005	**1.82 ± 0.2**	0.98 ± 0.12	0.33 ± 0.04
035	0.12 ± 0.00	0.18 ± 0.00	0.1 ± 0.00
CSS	0.92 ± 0.02	0.61 ± 0.04	0.3 ± 0.01
R(FI)	0.53 ± 0.06	0.45 ± 0.02	0.37 ± 0.08
PD	1.44 ± 0.12	0.75 ± 0.12	**0.95 ± 0.09**
CA	0.41 ± 0.04	0.27 ± 0.04	0.2 ± 0.02
MT	0.29 ± 0.03	0.18 ± 0.02	0.19 ± 0.04
ABR	0.42 ± 0.06	0.45 ± 0.6	0.14 ± 0.02
MDB	0.52 ± 0.02	0.5 ± 0.01	0.15 ± 0.00
RGT	0.09 ± 0.00	0.12 ± 0.00	0.09 ± 0.00
ID2	0.5 ± 0.04	0.43 ± 0.03	0.21 ± 0.02
ID4	0.55 ± 0.02	0.44 ± 0.04	0.17 ± 0.00
MZ	0.53 ± 0.04	0.18 ± 0.02	0.22 ± 0.02
MB	0.45 ± 0.04	0.64 ± 0.07	0.14 ± 0.02
GC	0.13 ± 0.01	0.18 ± 0.01	0.13 ± 0.00
GF	0.91 ± 0.02	0.65 ± 0.04	0.28 ± 0.01
SCO	0.83 ± 0.03	**1.24 ± 0.06**	0.85 ± 0.04
RC	1.03 ± 0.1	1.06 ± 0.08	0.64 ± 0.07
CFO	**3.63 ± 0.01**	**1.77 ± 0.01**	**1.03 ± 0.03**
BDG	0.4 ± 0.03	0.3 ± 0.02	0.2 ± 0.01
MRZ	0.08 ± 0.00	0.11 ± 0.00	nd
BDB	1.26 ± 0.02	0.63 ± 0.01	**0.92 ± 0.02**
MDG	**1.5 ± 0.00**	0.94 ± 0.00	**0.9 ± 0.01**

***Table 4**: Quercetin derivatives levels in apple peel extracts (mg/g DW). Data are the mean ± SD of three biological replicates. (nd = not detected)*

This class of flavonoids turned to be almost exclusively localized in peel, as shown in the chromatographic profile of AB peel extract in fig 47), in flesh extracts only traces of them were identified (< 0.05 mg/g DW) and only in few out of the 37 cultivars analyzed, including the reference ones.

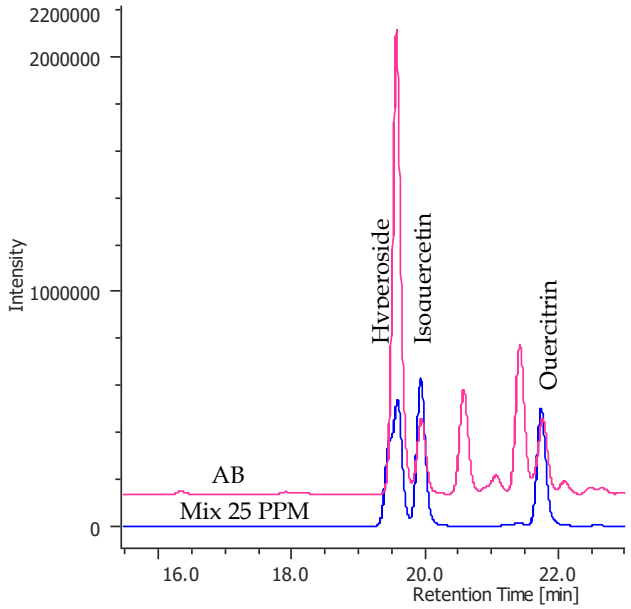

Figure 47: Overlay of chromatographic profiles (wavelength 360 nm) of the standard mix and free peel extracts of AB sample.

Hyperoside is one of the major flavonoid found in apple fruits peel, and can even be considered as a marker for apple peel [64,76]. Besides it, several studies have identified other quercetin derivatives in the apple peel, as well as other flavonols, like isorhamnetin and kaempferol derivatives [16,42,64], which have not been here identified. Our results are consistent with those of previous studies, which reported hyperoside and quercitrin as the predominant compounds, followed by isoquercetin and additional flavonol glycosides [46,76]. The concentration of quercetin derivatives found in this work is in line with results by Tsao et al. (2003) [52], who reported levels between 1.0 and 2.0 mg/g DW in reference cultivars, similarly to Jacobek (2013) [16]. Evidence demonstrating that quercetin and its derivatives have enormous therapeutic potential in prevention and treatment of several chronic diseases, including cardiovascular and neurodegenerative diseases, as well as cancer, is increasing [77]. The antitumoral activity, which has been demonstrated both in vitro and in animal models, occurs through several mechanisms, starting from the scavenging activity against free radicals [77], up to the regulation of cell cycle signaling, and pro-apoptotic properties [78].

In addition, an important protective role of quercetin and its glycosides has been reported for neurodegenerative diseases. Their neuroprotective effects are mainly due to their capacity of up- or down-regulating genes involved in cellular responses such as expression, proliferation, protein secretion and inflammatory response [79]. As far as the comparison between the 35 ancient varieties taken into consideration and the commercial ones, the gap in terms of polyphenol concentration is, in some cases, very marked. In fact, the latter are often in a lower range, and share a fairly overlapping polyphenolic pattern, which is obviously the result of the long-standing breeding process, that has brought on the market varieties with a precise organoleptic and nutritional profile. Among the ancient apples there is surely a great variability in the polyphenolic pattern, as it is strongly influenced by the genotype. There are varieties with an evident prevalence of phenolic acids and catechin (Gris Canaviot, Garola) which are lower in other identified compounds, in particular flavonols. On the contrary, cultivars with high quercetin derivatives concentrations (such as Commercio (FO) and Arkansas Black), are not particularly rich in caffeic acid derivatives. Our data have often proved to be consistent with what was found in the literature; however, it is often hazardous to compare results obtained through different extraction and analytical protocols, even more on different apple genotypes. The differences found with other authors are due to several factors, primarily the sampling method, which has a great Impact on the final results. In many cases samples are not immediately frozen with liquid nitrogen. This fast cooling process involves a uniform distribution of small ice crystals, preserving the characteristics of the product and preventing the degradation of polyphenols [80]. Another critical point that can affect the extraction yield and the quantification of compounds, is the extraction process; raw plant extracts are often ultrasonicated to release as many active compounds as possible, destroying cell structures in which they are contained [81].

In vitro antioxidant activity

The antioxidant capacity (AC) of apple peel and flesh free extracts was assessed through two different *in vitro* assays i.e., ABTS and ORAC. Since up to now there is not yet an official standardized assay for measuring the **total antioxidant power** of a sample, it is good practice to apply more than one method, since the measured antioxidant capacity of a sample depends on which free radical generator or oxidant are being used in the

measurements, and especially the variety of molecules present In raw extracts react in different ways [82]. The results of AC obtained by the ABTS test on the flesh (darker bars) and peel (lighter bars) extracts are shown in fig. 48. Bars were superimposed to highlight the clear difference in antioxidant capacity, of the two analyzed apple tissues.

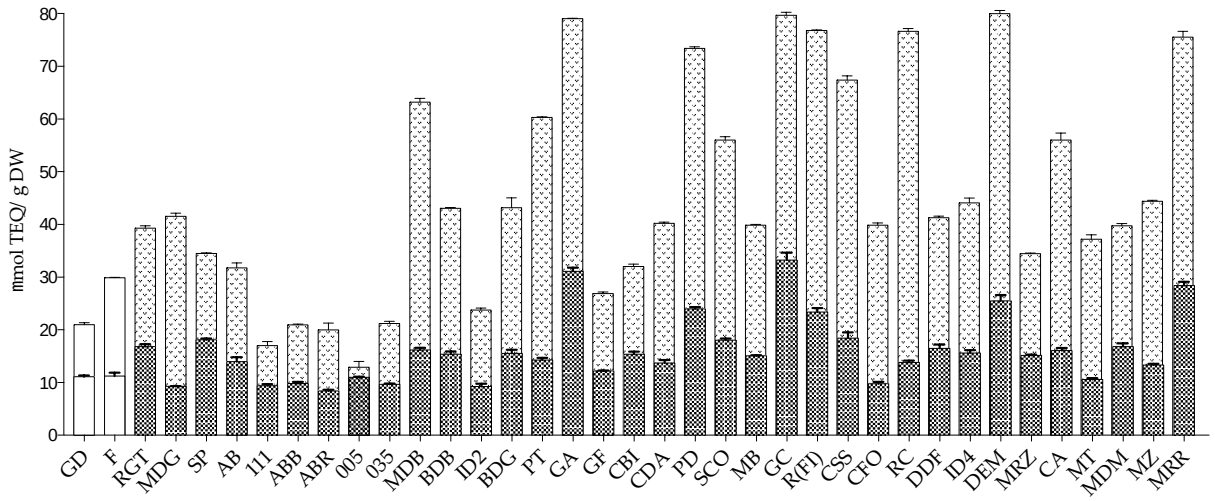

Figure 48: Antioxidant capacity, measured with ABTS assays in apple peel (lighter bars) and flesh (darker bars) extracts. Data are expressed as µmol TE/g DW) and each value represents the mean ± SD.

As for the antioxidant activity of **flesh** extracts, the activity ranged from a minimum of 8.0 to a maximum of 33.0 µmol TE/g DW. Reference cultivars, GD and F, had a value slightly higher than 10 µmol TEq/g DW, and most of ancient cultivars showed an AC within this range. Some cultivars, like PD, R(FI), DEM and MRR turned more powerful as antioxidants compared to commercial varieties, reaching a Trolox Equivalent capacity more than double. Gris Canaviot and Garola were the two cultivars with the highest antioxidant activity into the flesh, reaching 33.0 µmol TEq/g DW, while the lowest activity, below 9.50 µmol TEq/g DW, was found in flesh extracts of cultivars 111, Mela del Giappone, Abbondanza Rossa and Idice 2. The antioxidant power of **peel** extracts has been found in a range between 12.0 and 76.0 µmol TEq/g DW. Looking at the graph shown in fig. 48 we can clearly notice at a first glance that the antioxidant power of peel extracts was almost double or triple than flesh ones, even if the pattern is quite uneven, also for the reference cultivars. In fact, by comparing the results of the antioxidant activity of the two tissues, the peel is confirmed as higher than the flesh in terms of capacity and this trend is confirmed for all cultivars analyzed in this study.

As for the reference fruits, there is a marked difference between the tissues of Fuji than those of Golden Delicious, in fact the ratio peel/flesh is respectively 2.52 and 1.79. As far as ancient fruits are concerned, most of them are in the range of commercial varieties, while some of them, such as Pum Tosc, Mela del Giappone and Rus Canaviot, reach ratios between 4.0 and 5.0. On the contrary there are two varieties with a ratio even lower than that of GD, and they are 111 and 005, in fact for the latter the antioxidant activity of the two tissues is almost completely superimposable (ratios equal to 1.7 and 1.1 respectively). From the graph there are three distinct groups: the ancient varieties similar to Golden Delicious, corresponding to five cultivars, with the lowest antioxidant capacity, between 16.0 and 20.0 µmol TEq/g DW, those with activity equal to Fuji i.e. three cultivars, in the range between 25.0 and 37.0 µmol TEq/g DW, and the remaining varieties, that account for the majority, with instead higher values. Among the latter, certainly stand out, in increasing order of antioxidant activity: Belfiore di Trento (PD), Mela Rosa Romana, Rosa, Rus Canaviot, Garola, Gris Canaviot and Democrat.

As concern results of antioxidant activity, measured with the ORAC assay, both for apple peel (darker bars) and for apple flesh (lighter bars) are reported in fig. 49.

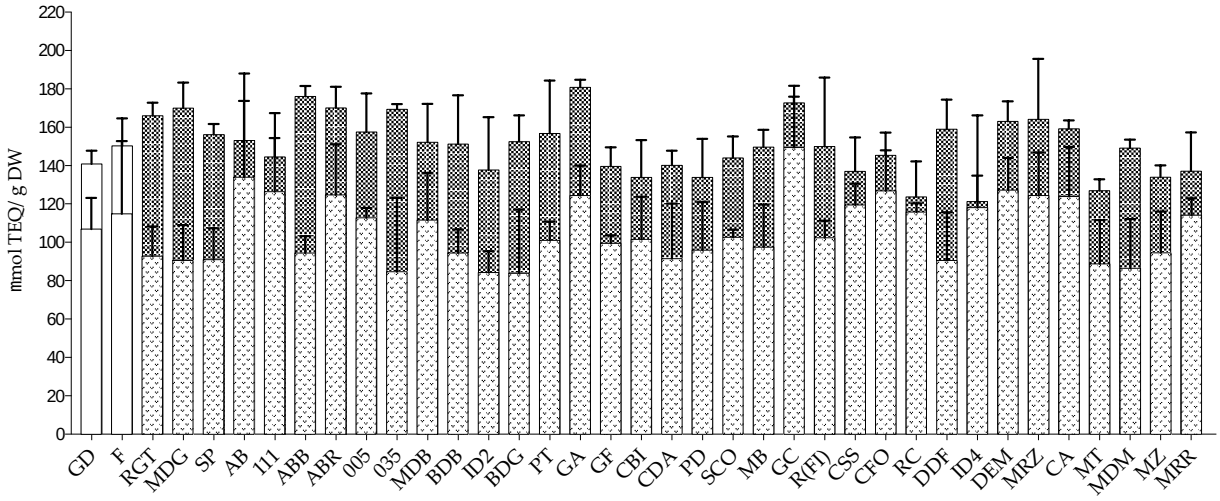

Figure 49: Antioxidant capacity, measured with ORAC assays (µmol TEq/g DW) in apple peel (lighter bars) and flesh (darker bars) extracts. Data are the mean ± SD.

It is clear that antioxidant capacity of apple **peel** was confirmed to be higher than those of flesh; indeed, the antioxidant power of **flesh** extracts was between 85.0 and 150.0 µmol TEq/g DW, while that of peels were between 120.0 and 180.0 µmol TEq/g DW.

In case of ORAC, results were much more homogeneous among samples, showing fewer differences compared to those emerging from the ABTS one. This consideration also applies to the peel/flesh ratios between ORAC results, which are also similar among the reference cultivars (both equal to 1.4), and among the ancient cultivars that are in a range between 1 and 2. Despite this, it remains evident, for some of them, the ratio between peel and flesh, as in the case of 035, Abbondanza and Mela del Giappone equal to the double in advantage of the peel.

The results here obtained show that the ratio in antioxidant capacity between peel and flesh was similar to what reported by Kschonshek (2018) [64], in fact the most promising tissue was found to be the peel with a ratio of 3.5-3 in favor of the latter depending on the assay used (ORAC and ABTS respectively), and the reference apple (Golden Delicious) was always lower than the old varieties. Comparably to our results, activity measured with ORAC assay showed fewer differences, both at tissue and cultivar level. The ratio found by Wang (2015), using ABTS assay, between antioxidant capacity of peel/flesh was much higher than ours, but still confirms the marked activity of the peel at the expense of the flesh. This author also reported a marked difference in the antioxidant capacity between fruits of **four** wild cultivars derived from the red flesh *Malus niedzwetzkyana* Dieck, and those of the white flesh references ones (Gale Gala and Golden Delicious), the former having a higher AC compared to the latter, and authors concluded that this was related to the different content in anthocyanins. Among the apple cultivars studied in this work, two of them i.e. Abbondanza (ABB) and Abbondanza rossa (ABR) differ with each other for the flesh color (white and red, respectively), but our results did not show differences in their antioxidant capacity, neither in the flesh, nor in the peel (Fig. 48, 49).

In light of the results of antioxidant capacity obtained with these two different assays, it clearly emerges that some ancient cultivars are definitely recognized as more active than others. In particular Gris Canaviot and Garola, for which both tissues analyzed reach the highest levels of antioxidant capacity. A positive correlation has been found between the total polyphenol content and the results of the ABTS assay on flesh extracts (r Pearson coefficients 0.96), while it was lower with regard to ORAC (r Pearson coefficients 0.55); a similar correlation was also found by others between the total content of polyphenols (both in peel and in flesh) and the results of antioxidant activity measured by different

assays (DPPH, FRAP-FZ) [17,49,83]. In addition, both chlorogenic and caffeic acid were correlated with results of ABTS assay (r Pearson coefficients 0.76 and 0.87 respectively) and ORAC assay (r Pearson coefficients 0.66 and 0.63 respectively) in flesh extracts. These results are in line with what has been reported by other authors, where it emerges that chlorogenic acid and some flavonoids contribute largely to the antioxidant capacity of apple fruit extracts [15,84]. Other studies, however, report weaker correlations between hydroxycinnamic acids and antioxidant activity, showing how flavan-3-ols (monomers, dimers, and oligomers) and phloridzin are instead crucial for it [42,83].

Principal Component Analysis (PCA) of data

The concentration of each phenolic compound identified by chromatographic technique, the total polyphenol and flavonoid contents and antioxidant capacity in the two tissues were subjected to the Principal Component Analysis (PCA) with the aim of identifying similarities and differences between apple cultivars analyzed. This explorative analysis was used to select the most diverse cultivars, to be further assessed for their biological activity. PCA of peel extracts (fig. 50A) shows that the first two principal components (PC1 and PC2) accounted for 66% of the total variability. The variables that mainly contributed to PC1 (40.8%) were antioxidant capacity, chlorogenic acid, phloridzin and catechin levels. The PC2 (25.2%) was associated with total polyphenol and flavonoid content, and epicatechin. Some cultivar formed a compact cluster in the quarter I. In the quarter IV, cultivars with a greater antioxidant activity are found, and the most diverse ones are the following: MRZ, ABB, GD, 5 (quarter I); 111, GF, ABR, MdM (quarter II); GC and DEM (quarter III) and R(FI), RC, PD (quarter IV). The multivariate analysis of data referring to the chemical composition and antioxidant capacity of flesh extracts is shown in fig. 50B. At a first glance, it appears that cultivars seem to be more homogeneous in the flesh composition, if compared to the peel. The first two principal components accounted for 74.2% of the total variability. The variables that mainly contributed to PC1 (54.9%) were quercetin derivatives (isoquercetin and hyperoside), while PC2 (19.4%) was mainly associated with quercitrin and catechin. Based on the flesh composition, the most diverse cultivars turn out to be: MZ, GD (quarter I); SP, RGT, ABB (quarter II); R(FI), GC, GA (quarter III); BdG, MRR, MT (quarter IV).

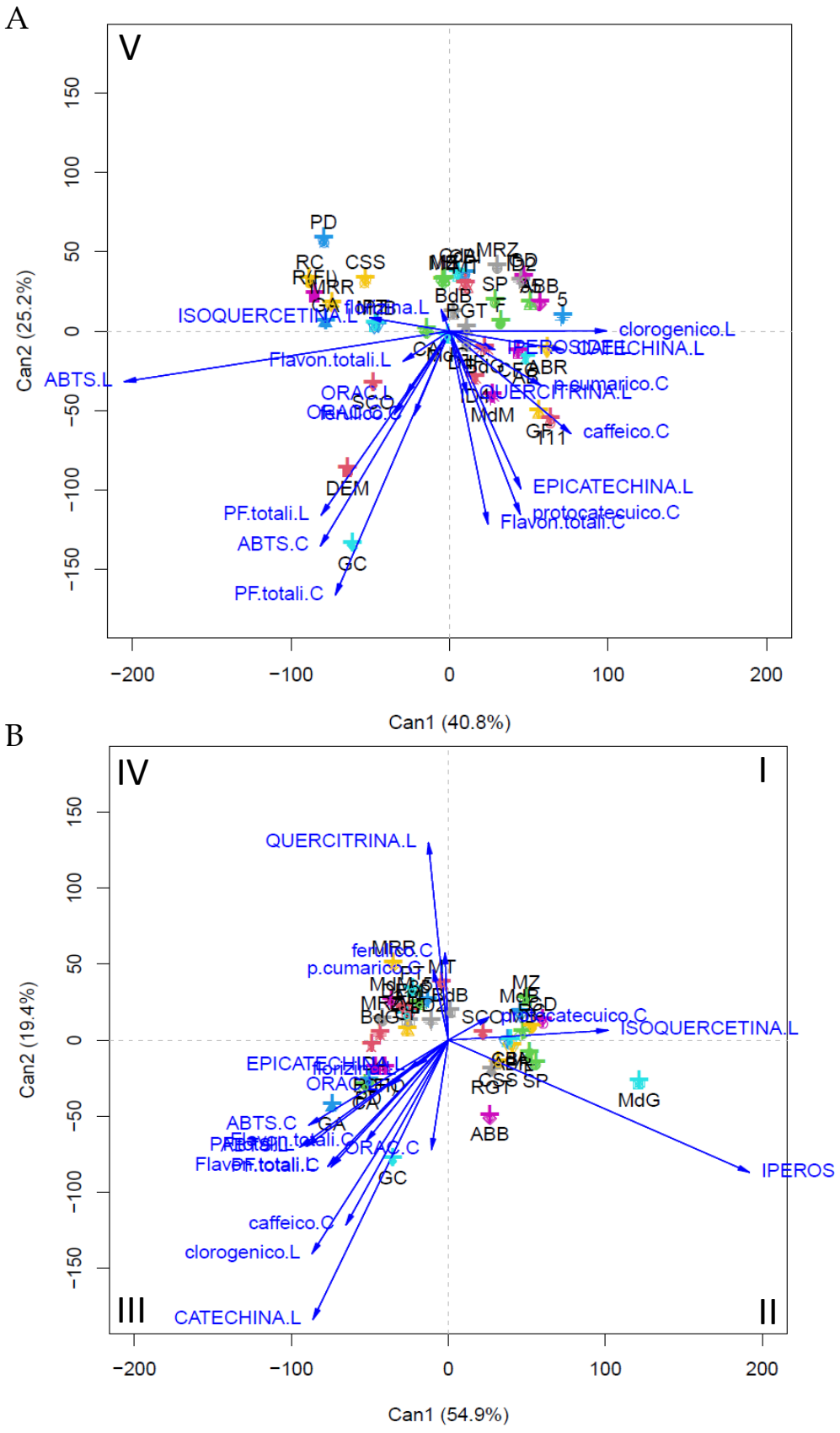

Figure 50: Canonical discriminant analysis of the antioxidant activity (ORAC and ABTS) and the phenolic acids and flavonoids content in apple peel (A) and flesh (B) extracts. Can1 = canonical component 1; Can2 = canonical component 2.

Apple can be a valuable fruit for the production of nutraceuticals and its beneficial effects are related, more than to a specific compound, to the whole **phytocomplex**. The genesis of many non-communicable diseases, which include cancer, heart disease, stroke, diabetes, neurodegenerative diseases, involves multiple steps with signaling pathways that may be sensitive to different molecules, and that can be better targeted by a combination of bioactive compounds in a natural extract, rather than by a single purified component [85]. Thus, a last multivariate analysis has been produced taking into account both tissues, the values of total polyphenols and flavonoids and the total indices (intended as the sum of individual compounds) of polyphenols and flavonoids quantified by HPLC (data not shown). From the graph reported in fig. 51 it is evident how much more homogeneous the data of the flesh extracts (indicated by the letter "P" before the name of the cultivar) are compared to the peel (indicated by the letter "B" before the name of the cultivar), which certainly show more variability. Total flavonoid content contributed more to PC2 (31.5%), while total flavonoid index was mainly related to PC1 (58.2%).

Therefore, in light of these results, the following cultivars were chosen for the flesh: **ABR, ABB, MDG, 35, 5, 111, GC, GD**; while for the peel: **GD, DEM, GC, SCO, RGT, 35, 5, 111, AB, PD, CFO, ABB**.

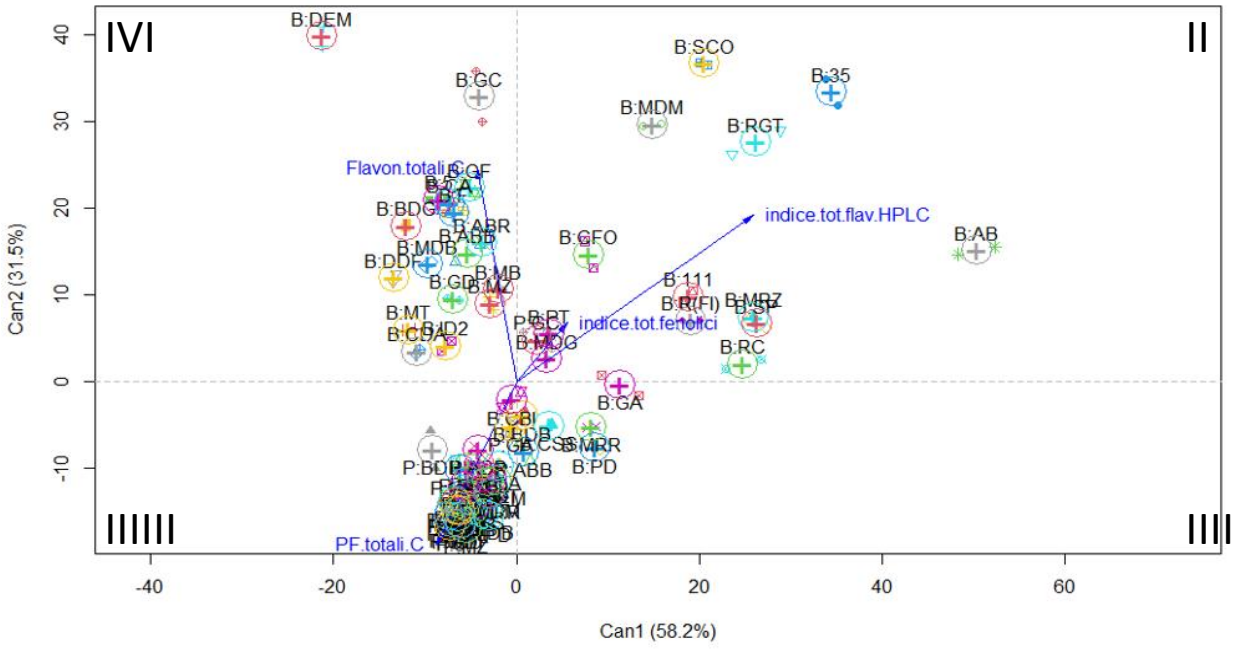

Figure 51: Canonical discriminant analysis of total polyphenols and flavonoids and the total indices (intended as the sum of individual compounds) of polyphenols and flavonoids quantified by HPLC in apple peel (indica ted by letter "B" before abbreviation of the cultivar) and flesh (indicated by letter "P" before abbreviation of the cultivar) extracts. Can1 = canonical component 1; Can2 = canonical component 2.

Conclusions

This study provided a survey of the phytochemical profile and antioxidant capacity of 35 Italian ancient cultivars of apple, in comparison with two widely consumed commercial cultivar, Golden Delicious and Fuji. Our data confirm that apple fruits are a valuable source of bioactive phenolic compounds, both in flesh and in peel, and also highlight their different localization in the two tissues, with phenolic acids mainly present in the flesh, while flavonols and dihydrochalcones mainly present into the peel. This is an interesting fact, because it places a fixed point in the "*peeling or not*" issue of apples; it is clear that discarding this part of the fruit would cause a significant loss of bioactive compounds. However, this reasoning must be scaled down, taking into account that apple peel only represents 10% of the whole fruit. In addition, the *in vitro* antioxidant assays have allowed to focus better on the nutraceutical properties of individual cultivars. Results show a wide variability in the polyphenolic profile among the different genotypes, and confirm that, on overall, ancient cultivars turned richer than the commercial varieties for most identified compounds, with some varieties, such as Gris Canaviot, Garola, Democrat, and Mela Rosa Romana reaching very high levels of polyphenols and antioxidant capacity. This confirms the great nutraceutical potential of the ancient cultivars, since health-beneficial effects have been recognized for these specialized metabolites. Undoubtedly, this added value deriving from the great abundance in bioactive compounds is counterbalanced by a bitter taste, a higher astringency and a faster enzymatic browning, all characters nowadays absent in the most widely consumed commercial varieties. Considering our results, it is clear that these local varieties need to be preserved and re-evaluated for several reasons: first of all, they can be used for breeding purposes to develop new varieties with enriched nutraceutical traits, or as a raw material to develop supplements endowed with health beneficial effects. Moreover, the conservation of the ancient apple varieties can contribute to maintain the biodiversity in the territories where they are cultivated, where they became part of the historical and cultural heritage of local populations. The phytochemical screening on the ancient apple cultivars allowed to Identify the cultivars that mostly differ with each other's. These will be further investigated for their capacity of preventing and/or treating some type of cancers. Since several Italian varieties (**Melannurca** and **Pelingo apple**) have shown strong *in vitro* antiproliferative activity against breast cancer

cell lines, correlated with their polyphenolic pattern [25,29], the ancient apples objects of our study will be investigated for their chemopreventive and antiproliferative potential, laying the basis for the development of functional foods, food supplements or therapeutic products.

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Chenopodium quinoa Willd.

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Free and Conjugated Phenolic Profiles and Antioxidant Activity in Quinoa Seeds and Their Relationship with Genotype and Environment

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Quinoa: general information and nutraceutical profile

Quinoa (*Chenopodium quinoa* Willd.) seeds have an excellent nutritional profile [1,2], due to their remarkably high protein content, well-balanced content of essential amino acids comparable, in terms of quality, to that of casein [3], and the presence of dietary fiber, vitamins, and unsaturated fatty acids, e.g., linoleic and α-linolenic acids [2–6]. Quinoa seeds are considered an excellent example of a "functional food", able to exert health-promoting effects also due to the presence of secondary metabolites [6]. These include phenolic acids and flavonoids [7], as well as terpenoids, steroids, and large amounts of α- and γ-tocopherol, which are known antioxidants [8]. For some of these specialized metabolites, a clear role in preventing various human diseases, such as neurological and other chronic disorders, has been recognized, as a result of their effects on cell signalling and metabolism [6,9,10].

Figure 52: Chenopodium quinoa Willd

Quinoa cultivation remained, for a long time, exclusive to Andean populations, especially from Bolivia and Peru [9], and, to a lesser extent, from Chile and Ecuador. The wide genetic diversity of this species, resulting from its fragmented and localized cultivation over the centuries in the Andean region, together with its high tolerance to extreme environmental conditions and abiotic stresses [10,11], has led to the differentiation of five major ecotypes, based on their ability to adapt to specific agroecological conditions [9]. Nevertheless, in recent decades, with the boom in demand for "**superfoods**", the interest in this ancient Andean halophytic seed-producing crop has become global, and its cultivation has been

growing accordingly. In less than twenty years, the global demand for quinoa grew to such an extent that it led to the triplication of the Andean areas dedicated to its cultivation [12]. Moreover, the adaptability and stress tolerance of this plant is being exploited to establish cultivations outside the Andean territories [9,13]. As a consequence, the number of countries growing quinoa has rapidly risen starting from the 1980s, and, nowadays, more than 95 countries are cultivating or testing quinoa as a crop [14]. In the early 1990s, quinoa began to be cultivated in Europe, and the crop has been successfully tested in several Mediterranean countries, such as Greece, Morocco, Spain, and Italy [15–18].

The main concern about the introduction of this crop was related to its high sensitivity to photoperiods during the reproductive phase [19]. The best results were achieved with the sea level/coastal ecotype from central and southern Chile, since it is the best adapted to temperate environments. Using these Chilean quinoa lines, a new variety, called Titicaca, was bred in Denmark [20]. Titicaca is one of four European quinoa cultivars (with Puno, Jessie, and Zeno) originating from different gene pools and already tested in various countries, including Germany [21] and Italy, where several field trials have been carried out at different latitudes to test the adaptability of the crop to varying environments [21,23,24]. Pulvento et al. [22] reported good plant performance and tolerance to high temperatures and water deficit, typical conditions of southern Italy, for cultivars Regalona-Baer and Titicaca. Several studies point to the importance of environmental and/or agronomical factors in affecting the nutritional properties of quinoa. For example, Reguera et al. [23] showed that amino acid profiles, total protein content, mineral composition, and phytate content varied in seeds of three cultivars (Salcedo-INIA, Titicaca, Regalona) grown in three different countries (Spain, Peru, and Chile). However, the impact of agroecological conditions and crop management techniques on quinoa seed quality in terms of functional bioactive compounds (e.g., phenolics) has been little explored. Miranda et al. [24] reported that seeds of several quinoa genotypes grown in different geographical locations in Chile contained variable amounts of phenolics and flavonoids. Considering that these secondary metabolites are plastically produced in plant tissues as an acclimation response to environmental cues, their concentrations in plant tissues are the combined result of several factors, including genetic components, environmental conditions, and the complex interplay between the two [25–27].

In the present work, we checked this hypothesis by analyzing the phenolic profiles of quinoa seeds of the same variety from different agroecological environments and verified if the antioxidant activity (AA) of seed extracts likewise changed. Such analyses are useful to identify the environmental conditions that modulate the health-promoting characteristics of quinoa seeds. To this purpose, we evaluated the composition in bioactive free and soluble-conjugated phenolic acids and flavonoids and the AA of seeds of two quinoa varieties, the Chilean Regalona-Baer and the Danish-bred Titicaca, grown in northern Italy and compared them with those of seeds of the same varieties grown in Chile and Denmark. The present results could help to establish the best conditions (genotype/geographical cultivation zone) leading to seed enrichment in functional compounds and to reinforce the notion that good-quality quinoa can be grown in Italy.

Plant material and extraction procedure

Seeds of quinoa cultivars Regalona-Baer (opaque white) and Titicaca (pale yellow) harvested in 2017 were used. Seeds of cv. Regalona collected from plants grown in Italy (RI) were purchased from Dall'Ara & Lolli farm (Campiano, Ravenna, Italy; 44°18′10″ N, 12°12′07″ E; mean annual temperature 14.4 °C, max 19.0 °C, min 8.3 °C; total annual precipitation 646.0 mm), while seeds of the same cultivar grown in Chile (RC) were purchased from the seed company Semillas Baer (Temuco, Chile; 38°44′23″ S, 72°36′00″ W; mean annual temperature 10.9 °C, max 17.3 °C, min 5.9 °C; total annual precipitation 1355.2 mm). Seeds of quinoa cv. Titicaca grown in Italy (TI) were kindly supplied by D. Vanuzzi (Tuttoquinoa) and collected from plants grown in Sale (Alessandria, Italy; 44°58′54″ N, 8°48′37″ E; mean annual temperature 13.5 °C, max 18.6 °C, min 8.5 °C; total annual precipitation 501.5 mm), while Titicaca seeds from plants grown in Denmark (TD) were kindly supplied by S. Jacobsen (Copenhagen, Denmark; 55°38′46″ N, 12°17′53″ E; mean annual temperature 8.9 °C, max 19.2 °C, min −3.4 °C; total annual precipitation 848.8 mm). Each seeds sample represented a pool of ten different plants from the same growing field.

The extractions were performed in triplicate, following the procedure described by Tang et al. [28], with slight modifications. Seeds were ground in a knife mill for 4 × 30 s (A11 basic, IKA Werke GmbH & Co. KG, Staufen, Germany) and in a mortar to obtain a fine and homogeneous powder. The seed powder was then subjected to the "coning and quartering" sampling procedure, and three technical replicates were carried out. A 3-g aliquot of fine powdered sample was transferred in a 50-mL tube, mixed with 10 mL of 70% MeOH acidified with HCl (0.1%, v/v), and kept on an orbital shaker (Duomax 1030, Heidolph Instruments, Schwalbach, Germany) at 200 rpm for 3h at RT; after a 15-min ultrasound extraction (Elma Schmidbauer GmbH, Singen, Germany), the mixture was centrifuged for 30 min at 4000 rpm, and the supernatant, containing all extractable compounds, was collected. The procedure was repeated twice, and supernatants were combined to form the crude extract (CE).

Chemicals and reagents

The following chemicals and reagents were purchased from Sigma-Aldrich Italia (Milan, Italy): Folin–Ciocalteu's phenol reagent, 6-hydroxyl-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS), AlCl3, NaNO2, FeCl2, FeCl3, FerroZine®, fluorescein, 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH), phosphoric acid (85–87%, w/w), hydrochloric acid (37%, w/w), monobasic sodium phosphate (>98%), sodium hydroxide beads (>98%), and HPLC-grade solvents. Pure standards of phenolic acids (4-hydroxybenzoic, gallic, caffeic, chlorogenic, ferulic, p-coumaric, sinapic, syringic, trans-cinnamic, and vanillic acids) and flavonoids (quercetin, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, kaempferol, kaempferol-3-O-rutinoside, catechin, epicatechin, daidzein, and genistein) were purchased from Extrasynthese (Genay Cedex, France). All standards (>99.5% purity in powder form) were prepared as stock solutions at 1 mg/mL in methanol and stored in the dark at −18 °C for less than three months.

Preparation of Free (F), Base-Hydrolysed (BH), and Acid-Hydrolysed (AH) soluble fractions

To obtain the free (F) fraction, the CE was evaporated to dryness, re-suspended with 2 mL of acidified water (pH=2), and subsequently extracted with 2 mL of a diethyl ether/ethyl acetate mixture (1:1, v/v) three times. The organic phases were merged, evaporated to dryness, re-suspended in 2 mL of 70% MeOH, filtered through 0.2-µm nylon syringe filters, and stored at −80 °C until analysis.

The aqueous phases were also pooled and subjected to base and acid hydrolyses to obtain the base-hydrolyzed (BH) and acid-hydrolyzed (AH) fractions, respectively. A 2-mL volume of 10 N NaOH was added to 8 mL of aqueous phase, and the mixture was stirred for 1 h under N_2 flow and brought to pH 2 with concentrated HCl. The resulting solution was then subjected to the extraction with diethyl ether/ethyl acetate mixture as previously described. The combined organic phases were evaporated to dryness, re-suspended with 2 mL of 70% MeOH, filtered through 0.2-µm nylon syringe filters, and stored at −80 °C until analysis.

The same volume of the aqueous phase was subjected to acid hydrolysis, by adding 1.6 mL of 12 N HCl, and the mixture was stirred for 1 h at 85 °C in a water bath. The resulting solution was extracted three times as previously described, and the combined organic phases were evaporated to dryness, re-suspended with 2 mL of 70% MeOH, filtered through 0.2-µm nylon syringe filters, and stored at −80 °C until analysis.

Spectrophotometric determination of total polyphenol and total flavonoid content

Spectrophotometric assays were carried out as previously described in Materials & Methods in section "*Malus domestica* Borkh. Ancient apple (*Malus domestica* Borkh.) cultivars as a source of chemopreventive substances: analysis of phytochemical profile and antioxidant capacity of peel and flesh".

HPLC-DAD Determination of phenolic compounds

The chromatographic method was adapted from Tang et al. [28], with some modifications. The extracts were injected into a Jasco (Tokyo, Japan) HPLC-DAD system, which consisted of a PU-4180 pump, an MD-4015 PDA detector, and an AS-4050 autosampler. The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reverse-phase column (100 × 3 mm I.D., 3.5 µm). The mobile phase was a mixture of solvent A (water/formic acid 95/5, v/v) and solvent B (methanol/acetonitrile 95/5, v/v), with a composition gradient ranging from 95% to 5% of solvent A and flowing at 0.7 mL/min. Injection volume was 20 µL for all determinations, and analyte detection was carried out with a diode array detector (DAD) by monitoring at 280, 329, and 360 nm. Quantification was performed with pure standards using calibration curves ranging between 1.25 and 30 µg mL^{-1} ($r2 \geq 0.9634$).

In vitro antioxidant activity assays

The ABTS assay was carried out essentially as described by Thaipong et al. [29]. After incubating 950 µL of 7.4 mM 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in methanol with either 50 µL of methanolic Trolox solution at different concentrations (0.05–1.00 mM), methanol (blank solution), or the diluted sample in the dark at RT for 2 h, the absorbance of the solution was read at 734 nm in the V630 spectrophotometer. Calibration curves were set up by plotting the discoloration ratio (i.e., [$Abs_{\text{without TX}}/Abs_{\text{with TX}}$] − 1) as

a function of Trolox concentration. The antioxidant capacity of the sample, expressed as Trolox equivalents (TEs), was calculated by interpolating with the calibration curve.

The oxygen radical absorbance capacity (**ORAC**) assay was carried out as previously described in Materials & Methods in section "*Prunus avium* L. - Characterization of New Sweet Cherry cultivars for their phenolic profile, antioxidant activity and neuroprotective potential." The ferric reducing antioxidant power-ferrozine (FRAP-FZ) assay was performed as described by Mandrone et al. (2015) [30].

Statistical analysis

Statistical analysis was performed using GraphPad (San Diego, CA, USA) Prism Software v. 5.0. Comparison among samples was conducted through one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and values of $p \leq 0.05$ were considered statistically significant. Two-way ANOVA was carried out to analyse the genotype x cultivation area interaction.

Results and discussion

Free and soluble-conjugated total phenolic content (TPC) and total flavonoid content (TFC) in quinoa seed extracts

Phenolic acids and flavonoids in seed extracts were quantified by analysing the free and soluble-conjugated forms (and not the insoluble ones) because they represent the fractions of dietary phenolic compounds that are more rapidly absorbed by the stomach and small intestine and for which important health benefits have been reported [31]. Total phenolic content (TPC) and total flavonoid content (TFC) were evaluated in extracts of the four seed samples enriched in the solvent-extractable (free; F) and in both the acid-hydrolysed (AH) and base-hydrolysed (BH) soluble-conjugated forms (figure 53). As concerns TPC (figure 53A), AH was the richest fraction, with levels reaching 1.7–1.8 mg GAE/g DW of seed powder, followed by the BH fraction (0.6–0.8 mg GAE/g DW), while much lower levels were observed in the free form (0.2–0.3 mg/g DW). In both the F and BH fractions, no significant differences in TPC were observed between RI, RC, TI, and TD. By contrast, the

Danish-grown Titicaca seeds (TD) had about twice the concentration of AH soluble-conjugated phenolics as compared with the same cultivar grown in Italy.

As regards Regalona, the TPC in this fraction (AH) was very similar in both Chilean- and Italian-grown seeds, and not significantly different from that of TD (figure 53A). This suggests that the conjugated form of soluble phenolics is mainly linked through ether bonds, such as the glycosidic one, which is acid hydrolysable. The distribution of TPC into the three fractions in the quinoa seeds analysed here is in line with that reported by Tang et al. [28] in a white seed quinoa, but not with that by Gómez-Caravaca et al. [32] for genotypes Kancholla and Witulla. These authors reported that most phenolic compounds present in their seed extracts were in the free form, but it should be underlined that these authors used a different analytical method, i.e., HPLC-DAD-ESI-TOF-MS, which could explain the discrepancies. Although Repo-Carrasco-Valencia et al. [7] observed a huge variation in the proportion of soluble (free and conjugated) phenolics (ranging from 7 to 61%), among the ten genotypes taken into consideration, we did not observe large differences in the total amounts of soluble phenolics between the two T and R varieties. It should be pointed out that the TPC in the AH fraction might be overestimated, since several authors [28,33] indicated that, under acid hydrolysis, the degradation of free sugars can give rise to furan derivatives, which react with the Folin–Ciocalteu's reagent.

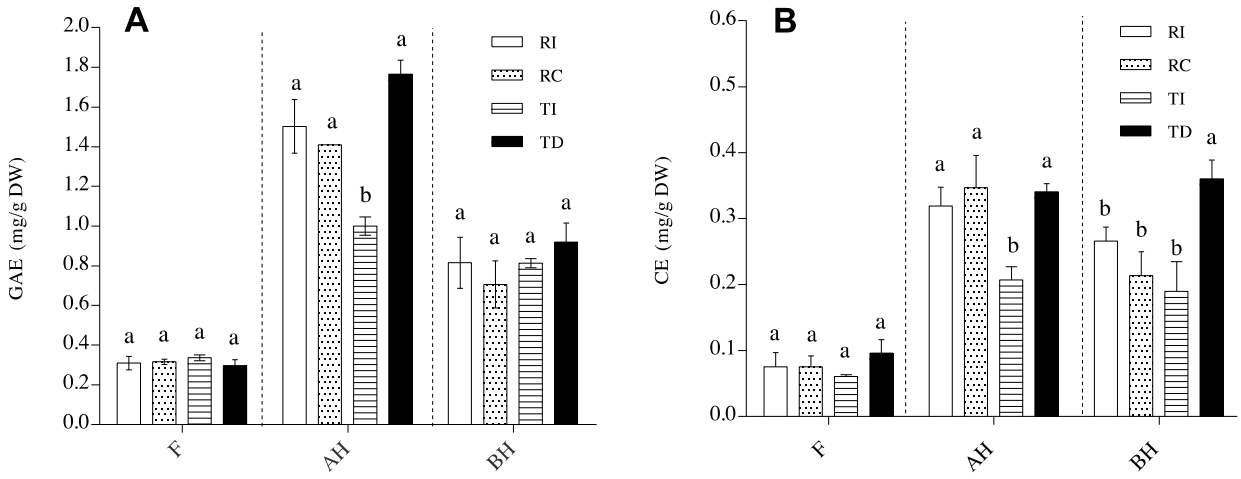

Figure 53: Total phenolic content (A) and total flavonoid content (B) in free (F), acid-hydrolyzed (AH), and base-hydrolyzed (BH) fractions of quinoa seed extracts. (A) TPC is expressed as mg gallic acid equivalents (GAE)/g DW seed powder. (B) TFC is expressed as catechin equivalents (CE)/g DW seed powder. RI: Regalona grown in Italy; RC: Regalona grown in Chile; TI: Titicaca grown in Italy; TD: Titicaca grown in Denmark. Data are the means ± standard error of two independent determinations with three biological replicates. Different letters within the same parameter indicate statistically significant differences at $p \leq 0.05$.

Thus, the distribution of phenolic compounds between the free and AH conjugated forms, resulting from the TPC determination, only provides a rough estimate, which must be confirmed through more specific analytical techniques.

As concerns TFC, both the relative distribution in the three forms and the pattern within each fraction were similar to those of TPC; the conjugated forms were more abundant than the free ones, even though, in this case, the levels of AH and BH soluble flavonoids were comparable (figure 53B). In plants, it is known that flavonoids are mostly conjugated to various types of molecules. The abundance of flavonoids in both the AH and BH fractions indicates that these were linked via both ether and ester bonds; in the former category, glycosides represent the most widespread form, while the latter suggest their linkage to organic acids and proteins, as demonstrated by Koistinen et al. [34]. As concerns the comparison among samples, the pattern of TFC in the free and AH fractions was very similar to that of TPC, with AH forms again higher in TD than in TI. Danish-grown Titicaca showed the highest TFC in the BH fraction, while no differences were found between the other three samples (figure 53B). Based on these results, both genotype and environmental conditions seem to exert their effect mainly on the conjugated form of phenolics, leaving the free form almost unchanged. Moreover, they indicate that TI had the lowest level of phenolics conjugated through ether linkages.

Chromatographic Analysis of Free and Soluble-Conjugated Phenolic Compounds in Quinoa Seed Extracts

The three fractions were subjected to high-performance liquid chromatography-diode array detector (HPLC-DAD) analysis to identify and quantify single phenolic compounds. These include phenolic acids (both hydroxycinnamic and dihydroxybenzoic acids), flavonols, flavan-3-ols, and isoflavones (figure 54).

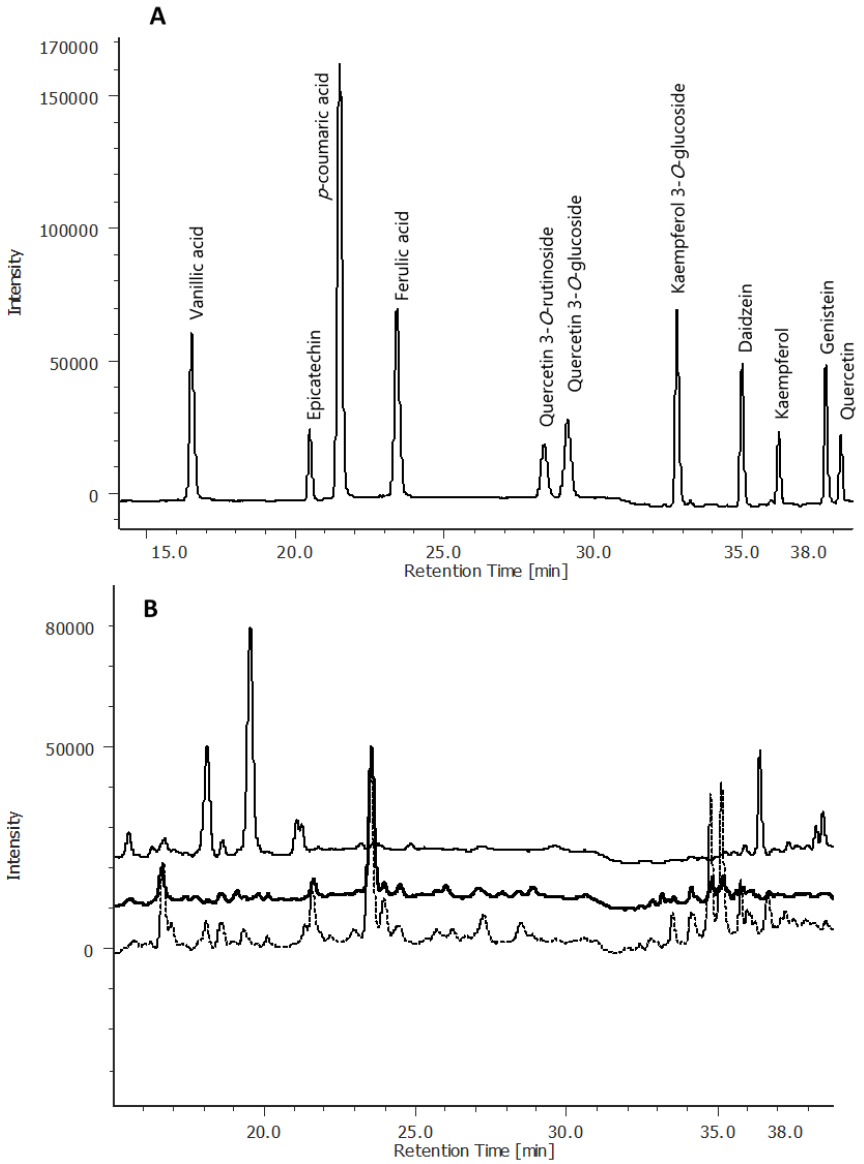

Figure 54: Chromatogram of HPLC-DAD separation of (A) standard phenolic acids and flavonoids, and (B) representative chromatographic profiles of free (upper line), base-hydrolyzed (middle line), and acid-hydrolyzed (bottom line) fractions of one seed extract (TI)

In the free fraction, vanillic and ferulic acids were the major hydroxycinnamic acids detected in all samples, the former being about 10-fold more concentrated than the latter (figure 54). Seed extracts of Chilean Regalona had a vanillic acid content about twice that of the same genotype grown in Italy, and of both TD and TI, whose concentrations were very similar. As for ferulic acid levels, no differences were observed between the two cultivars, no matter where they were grown (figure 55).

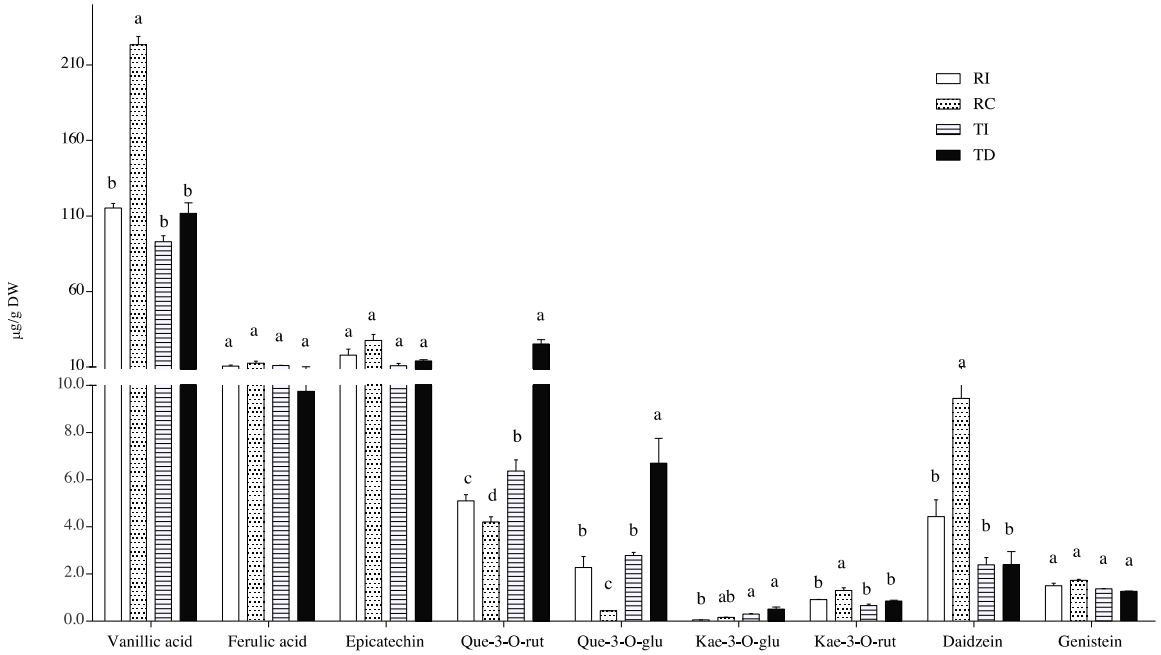

Figure 55: Free phenolic acid and flavonoid concentrations in quinoa seed extracts. RI: Regalona grown in Italy; RC: Regalona grown in Chile; TI: Titicaca grown in Italy; TD: Titicaca grown in Denmark. Data are the means ± standard error of two independent determinations with three biological replicates. Different letters within the same parameter indicate statistically significant differences at $p \leq 0.05$.

The prevalence of vanillic acid as the main representative of the phenolic acid class in the free form is in line with the results of Tang et al. [28] in a white seed quinoa, even though significant variations in the relative amounts of single phenolic acids in different quinoa genotypes and varieties were also reported by Repo-Carrasco-Valencia et al. [7]. Nevertheless, a comparison with the results from these authors is not easy, since they reported the soluble phenolic acid content without distinguishing between the free and the soluble-conjugated fraction.

As concerns flavonoids, epicatechin, quercetin, and kaempferol derivatives (quercetin-3-O-rutinoside, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, and kaempferol-3-O-rutinoside) and the isoflavones daidzein and genistein were detected in the free fraction (figure 55). Significant differences among samples were observed only in some cases. In particular, TD seeds showed higher levels of quercetin-3-O-glucoside and quercetin-3-O-rutinoside compared to seeds of the same cultivar grown in Italy (two- and four-fold, respectively), and compared to both Regalona extracts. In Regalona seeds, a higher level of these two quercetin derivatives was found in Italian samples as compared to Chilean ones.

Conversely, the latter showed the highest daidzein levels among all samples, and no differences in this isoflavone were found among other seeds. All seed extracts contained very low amounts of kaempferol derivatives, whose differences were of no biological relevance, nor were any differences observed as concerns genistein and epicatechin (figure 55). Thus, the agroclimatic conditions in which the Chilean Regalona seeds were produced seem to be particularly favourable for the accumulation of daidzein since, in this sample, the highest daidzein/genistein ratio (=6) was found. This value is very close to that observed by Lutz et al. [35] in some commercial quinoa seeds and suggests that, under certain environmental conditions, a seed with a good phytoestrogen-like activity can be obtained.

In the AH conjugated fraction, *p*-coumaric and ferulic acids did not vary considerably among samples (figure 56). The ferulic acid concentration was very similar to that found in the free fraction, while *p*-coumaric acid was not detected in the free form. As concerns flavonoids, the aglycones quercetin and kaempferol were found as the main representatives, and TD was confirmed to be the richest in these flavonols (about three-fold higher than in TI), while no differences were detected between the two Regalona samples (figure 56).

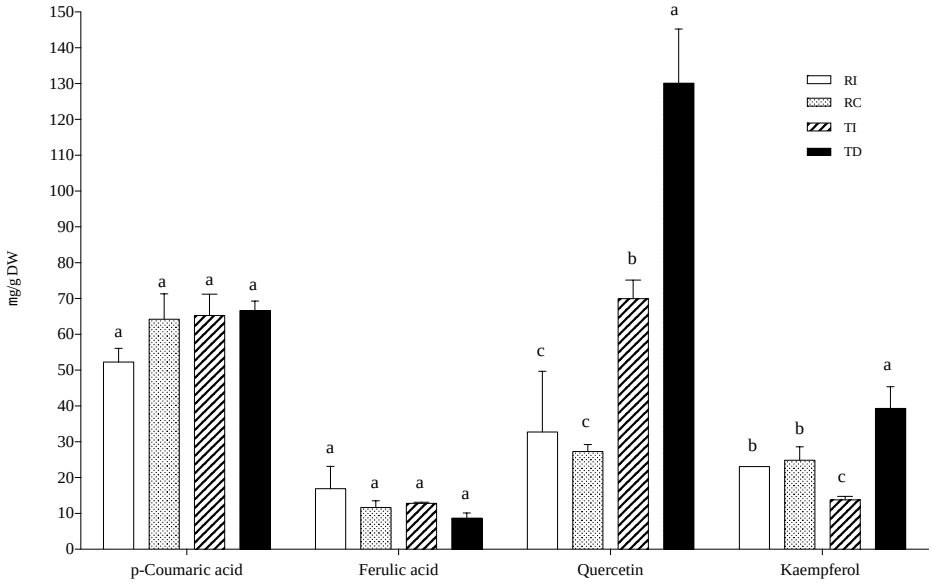

Figure 56: Phenolic acid and flavonoid concentrations in the acid-hydrolyzable soluble-conjugated fraction of quinoa seed extracts. RI: Regalona grown in Italy; RC: Regalona grown in Chile; TI: Titicaca grown in Italy; TD: Titicaca grown in Denmark. Data are the means ± standard error of two independent determinations with three biological replicates. Different letters within the same parameter indicate statistically significant differences at $p \leq 0.05$.

Phenolic acids and flavonoids were also detected in the BH soluble conjugated fraction. Both *p*-coumaric and ferulic acids were present, and the latter was detected at higher levels than in the AH fraction (figure 57).

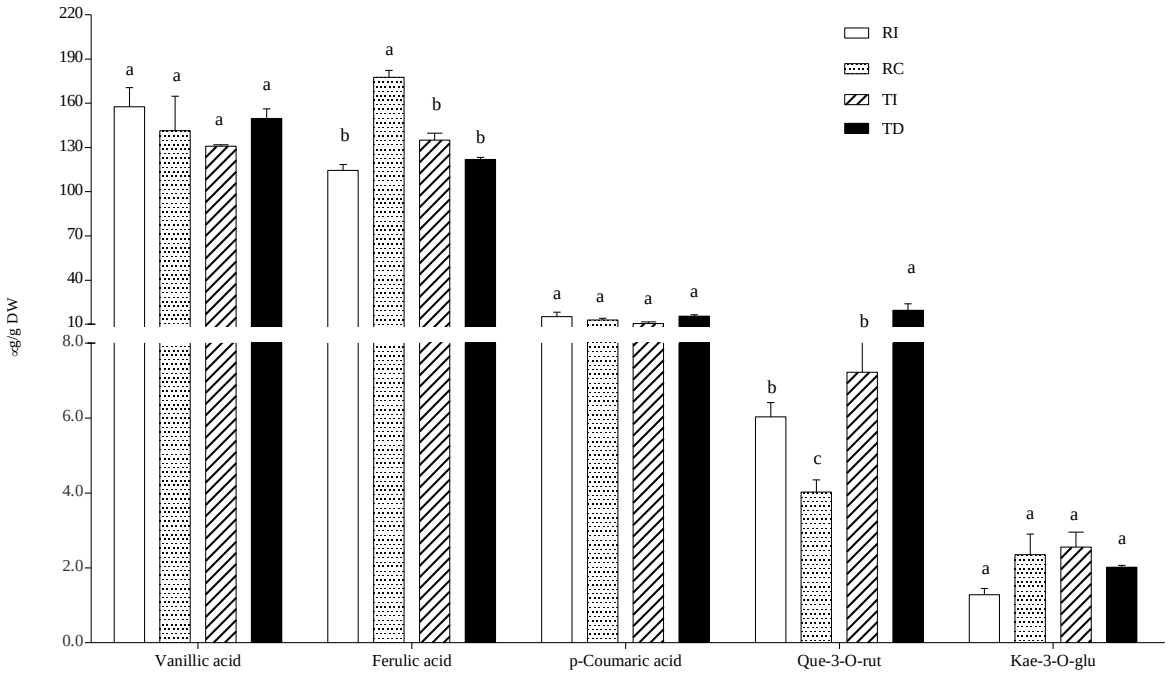

Figure 57: Phenolic acid and flavonoid concentrations in the base-hydrolyzed soluble conjugated fraction of quinoa seed extracts. RI: Regalona grown in Italy; RC: Regalona grown in Chile; TI: Titicaca grown in Italy; TD: Titicaca grown in Denmark. Data are the means ± standard error of two independent determinations with three biological replicates. Different letters within the same parameter indicate statistically significant differences at $p \leq 0.05$.

Vanillic acid was present at similar levels to those of the free fraction, while conjugated ferulic acid was over ten-fold more concentrated than in the free form. Thus, the results indicate that a relevant portion of these polyphenols is conjugated through ester bonds with soluble cellular components, such as short peptides and/or low-molecular weight oligosaccharides. It is known that ferulic and *p*-coumaric acids are covalently bound to mixed-linkage (1→3, 1→4)-β-D-glucans and to hemicelluloses, forming the soluble dietary fiber [36]. Being a dicot species, quinoa cell walls contain mainly xyloglucans in their matrix. The extractability of hemicelluloses during the preparation of crude seed extracts using sonication has been demonstrated [37] as well as the binding of ferulic acid to short-chain hemicelluloses [38]. Thus, the crude extracts used in this study probably contain soluble

feruloylated and/or coumaroylated xylo-oligosaccharides, which could explain the presence of high levels of aglycone components in our BH fraction.
Several health-promoting activities have been reported for these short-chain feruloylated oligosaccharides, including an immunomodulatory effect [39]. As concerns the comparison among samples, the results indicate that concentrations of vanillic and *p*-coumaric acids did not differ significantly between Regalona and Titicaca, no matter where they were grown, while for ferulic acid, a 55% higher concentration was found in RC compared to seeds of the same genotype grown in Italy; no differences were found between it and TD or TI seeds. Quercetin-3-*O*-rutinoside (or rutin) and kaempferol-3-*O*-glucoside were identified as the main flavonols in the BH fraction. As regards the former, the pattern was similar to those in the free and AH conjugated fractions, with DT being richer than the Italian-grown counterpart, while RI had a slightly, but significantly, higher rutin content than RC; no differences were observed for kaempferol-3-*O*-glucoside among the four samples (figure 57).

Some authors have investigated the phenolic composition of different quinoa varieties, and phenolic acids and flavonoids were reported to exist both in their aglycone and glycosidic forms [32,41,42]. On average, the concentrations of these compounds found in our work were similar to those found by other authors [40,41], but lower compared to other reports [32]. These discrepancies are not surprising, since several parameters were demonstrated to have a great impact on the extraction efficiency of these metabolites from plant material, such as sampling, solvent-to-solid ratio used for extraction, duration of extraction, and sonication treatment applied, among others [42]. The use of more advanced separative techniques, such as the HPLC-DAD-ESI-TOF-MS utilized by Gómez-Caravaca et al. [32], allowed these authors to identify more compounds, which are not detectable with other techniques, and this can also explain the differences.
Hirose et al. [43] reported that quinoa and buckwheat contain several quercetin and kaempferol derivatives, which are not present in any of the widely consumed cereals. They also compared the flavonoid pattern and *in vitro* antioxidant capacity of seeds cultivated in Japan with those of seeds from Bolivia and Peru.

Results indicated that the major differences between the two groups of seeds were found for quercetin and kaempferol derivatives, which were significantly higher in Japanese-grown seeds compared to South American ones, and this was accompanied by a significantly higher antioxidant capacity. Seasonal variations in the pattern of flavonol glycosides observed by these authors allowed them to hypothesize that sunlight was the factor that mostly influenced the accumulation of quercetin glycosides [43]. Thus, it is possible that variations in quercetin and its derivatives between Titicaca and Regalona grown at different latitudes may be due to differences in day length and light quality/intensity.

Even though the effect of latitude on flavonoid accumulation has yet to be fully clarified, due to the complexity of latitude itself as a parameter to be investigated [26], many studies have concluded that northern climates may have a positive impact on the biosynthesis of flavonoids in plants, although there are variations in the response among species and within individual flavonoid groups. Flavonols, especially moieties of quercetin or kaempferol, have been reported to accumulate in response to increased UV-B radiation [44], which typically characterizes the higher latitudes in the Northern Hemisphere. The prominent role for this subclass of flavonoids in regulating plant–environment interactions has been demonstrated, particularly as concerns the acclimation of plants to different light exposures [45]. In general, flavonoids may play prominent roles as scavengers of reactive oxygen species (ROS) generated by adverse conditions [46,47], and quercetin derivatives, due to their chemical characteristics, are particularly efficient in buffering alterations in ROS homeostasis [25]. Moreover, several reports indicate that they strongly affect phytohormone (auxin and ABA) signalling, due to their ability to affect the activity of a wide range of proteins [48,49]. Thus, the higher accumulation of quercetin derivatives in Titicaca seeds grown in Denmark compared to seeds of the same cultivar grown in Italy and to both Regalona samples might be the result of an acclimation mechanism to long light exposures, typical of the higher latitudes. The bioavailability and therapeutic potentials of quercetin and its derivatives in plant-based foods have been extensively investigated [50], and several studies demonstrated that they play relevant roles both in the prevention and treatment of chronic diseases, including cardiovascular and neurodegenerative ones and some types of cancer [51–53]. Thus, the enrichment of quinoa seeds in flavonol derivatives could represent an added value able to reinforce the well-known nutraceutical properties of this plant.

Antioxidant Activity of Quinoa Seed Extracts

The AA of the three fractions of Regalona and Titicaca seed extracts was evaluated using three different *in vitro* assays, i.e., FRAP, TEAC, and ORAC (figure 58).

The results obtained by the TEAC and ORAC assays univocally show that the fractions enriched in soluble-conjugated phenolic acids and flavonoids possess a higher AA compared to the free fraction (figure 58 A,B).

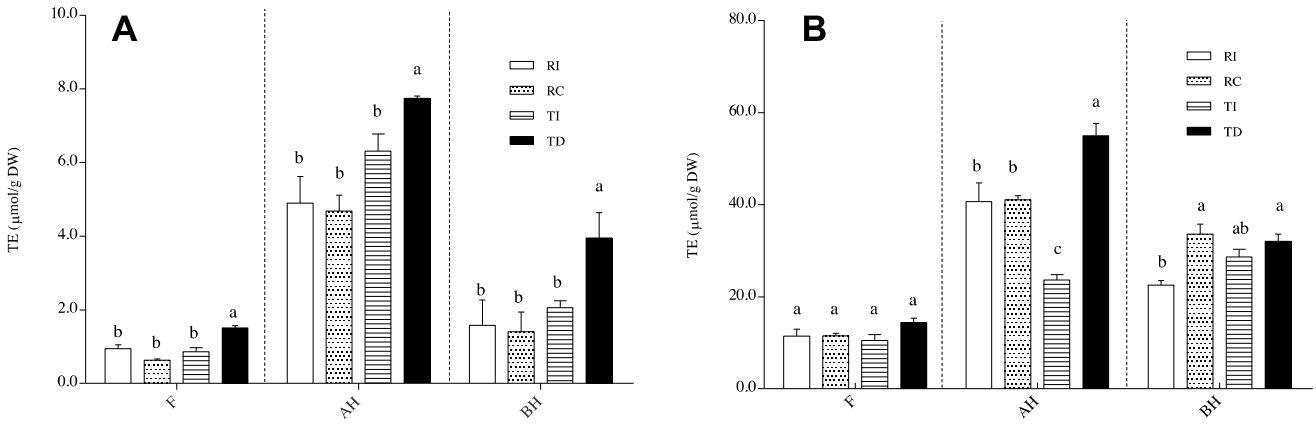

*Figure 58: Antioxidant activity, assayed by TEAC (**A**) and ORAC (**B**), in free (F), acid-hydrolyzed (AH), and base-hydrolyzed (BH) fractions of seed extracts from different quinoa samples. Results are expressed as Trolox equivalents (TE)/g DW seed powder. RI: Regalona grown in Italy; RC: Regalona grown in Chile; TI: Titicaca grown in Italy; TD: Titicaca grown in Denmark. Data are the means ± standard error of two independent determinations with three biological replicates. Different letters within the same parameter indicate statistically significant differences at $p \leq 0.05$.*

FRAP provided the same results (data not shown). Although this may derive, at least in part, from the above-cited overestimation due to the formation of 5-hydroxymethyl-2-furfural, which displays AA [33], it has been widely demonstrated that soluble-conjugated polyphenols and/or feruloylated/coumaroylated short oligosaccharides are able to greatly contribute to the antioxidant capacity of grains [54,55]. As concerns the comparison among samples, the results obtained with the TEAC/FRAP and ORAC assays are slightly different. For all fractions (free, AH, BH), the former assay (figure 58A; FRAP data not shown) revealed that TD had a slightly, but significantly, higher AA compared to TI and to both Regalona extracts, which, conversely, did not differ from each other. According to ORAC, no differences among samples were observed in the free fraction, while for the AH one, TD was confirmed to have the highest AA; no differences were found between the two Regalona samples grown in Chile and in Italy (figure 58B). In the BH fraction, a statistically significant difference emerged between the Italian-grown Regalona and both RC and TD,

even though the biological relevance of this difference is questionable. Thus, it can be concluded that the higher amount of flavonol derivatives in Danish-grown Titicaca might contribute to the higher antioxidant activity, given the strong redox capacity associated to this subgroup of flavonoids.

For an overall comparison among samples, the total phenolic index (TPI) and the total antioxidant index (TAI) were calculated as the sum of the individual phenolic compounds and AA, respectively, detected in all fractions.

As shown in figure 59A, despite a different distribution between the free and soluble conjugated forms, the results clearly indicate that the Chilean Regalona and the Danish Titicaca show a very similar TPI, while seeds of these cultivars grown in Italy respond to the different climatic/environmental conditions with a 20% lower accumulation of the main phenolic compounds compared to their counterparts.

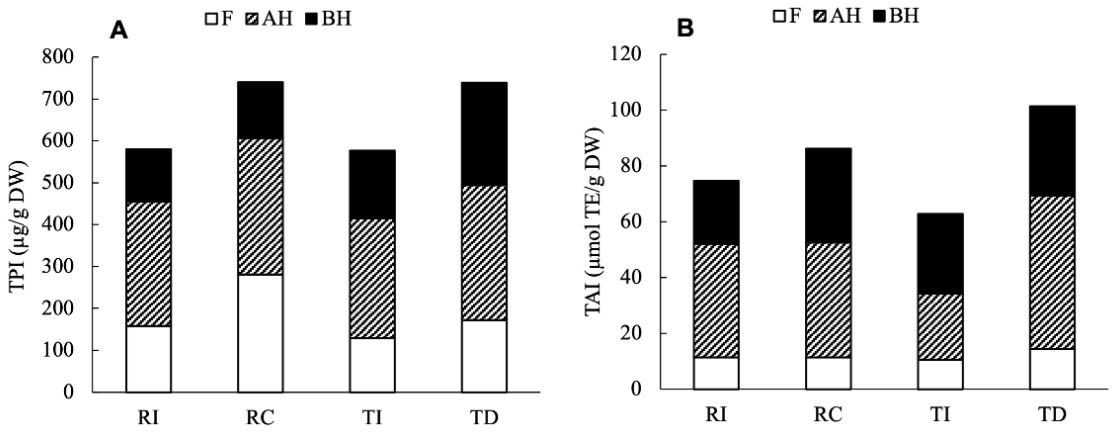

*Figure 59: Total phenolic index (TPI, **A**) and total antioxidant index (TAI, **B**) in the four seed extracts of quinoa. TPI was calculated as the sum of individual phenolic compounds detected, and TAI as the sum of ORAC-assayed AA in each fraction.*

The results of the two-way ANOVA indicate that the area of cultivation significantly contributed to the variance of TPI (78%, p = 0.005), while neither the genotypes nor their interaction had a significant impact on the variation (p = 0.13 and 0.56, respectively). As for the TAI resulting from the ORAC assay (figure 59B), it was only slightly lower (15%) in RI compared to RC, while a greater difference (40%) was observed between TD and TI; similar results were found for the TEAC assay (data not shown). These results confirm that the total antioxidant activity only weakly correlates with the TPI, as it is more tightly related to the specific composition in antioxidants; in fact, single compounds or classes of compounds can provide a greater contribution compared to others. The two-way ANOVA indicated that the total antioxidant index did not vary significantly depending on genotype (p = 0.61), while

the cultivation area, as well as its interaction with genotype, provided a significant contribution ($p < 0.0001$ and $p = 0.0031$, respectively).

Conclusions

In conclusion, the present results suggest that, both in terms of phenolic profiles and AA, genotype-dependent differences are not very relevant as far as Titicaca and Regalona are concerned, both of which are adapted to temperate climates, belong to the coastal ecotype, and were bred from gene pools originating from southern/central Chile. The results also indicate that agroecological conditions can, to some extent, alter these phytochemical profiles and the biological activities; in particular, light/UV-B intensity may have contributed to higher flavonol levels in seeds from Denmark [56,57]. Nonetheless, the changes are quite small and corroborate previous data [22], demonstrating that both Titicaca and Regalona can be successfully cultivated in Italy, where they maintain good growth, productivity, and nutritional properties.

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Abstract

During the internship period carried out at the Department of Organic Chemistry at *Universidad Autónoma de Madrid* (UAM), Madrid (ES), it was achieved the isolation of two pentacyclic triterpenoids, from an endemic Peruvian plant, *Jatropha macrantha* Müll. Arg., by applying of bio-guided fractionation technique. The two isolated molecules were subsequently identified as pomolic acid and euscaphic acid and have been proved to be non-cytotoxic on normal cells, while they have been shown to possess inhibitory activity *in vitro* against two transcription factors involved in cancerogenesis. The results of this research project have been published and will be included in the following essay.

Natural Product Research
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Triterpenoids isolated from *Jatropha macrantha* (Müll. Arg.) inhibit the NF-κB and HIF-1α pathways in tumour cells

Luis Apaza T. , Fabiana Antognoni , Giulia Potente & Ángel Rumbero Sánchez

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SHORT COMMUNICATION

Triterpenoids isolated from *Jatropha macrantha* (Müll. Arg.) inhibit the NF-κB and HIF-1α pathways in tumour cells

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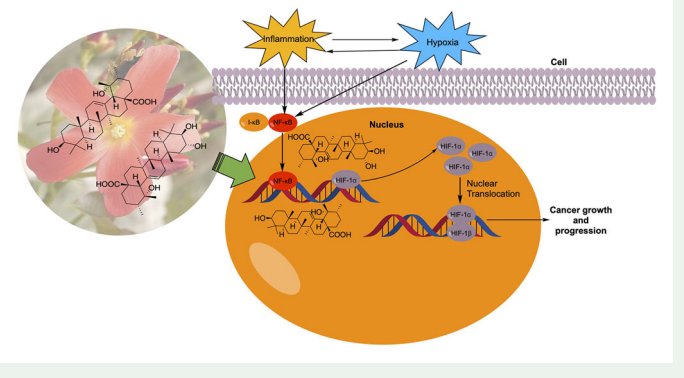
ABSTRACT

Activity-guided fractionations of *Jatropha macrantha* Müll. Arg. led to the isolation of pomolic acid (**1**) and euscaphic acid (**2**). The potential for inhibition against NF-κB and HIF-1α production of these two compounds was tested in different tumour cell lines. Compounds **1** and **2** showed an inhibitory activity of HIF-1α in the SK-MEL-28 (IC_{50}=3.01 ± 0.02 μM and 3.78 ± 0.02 μM), A549 (IC_{50}=9.97 ± 0.01 μM and 10.25 ± 0.01 μM) and U-373 MG (IC_{50}=6.34 ± 0.02 μM and 8.85 ± 0.02 μM) cell lines. In addition, compounds **1** and **2** showed an inhibitory activity on NF-κB in SK-MEL-28 (IC_{50}=1.05 ± 0.02 μM and 2.71 ± 0.01 μM), A549 (IC_{50}=3.63 ± 0.01 μM and 3.73 ± 0.02 μM) and U-373 MG (IC_{50}=2.55 ± 0.02 μM and 3.39 ± 0.01 μM) cell lines. This is the first report that isolates these compounds from *J. macrantha* and tests their antitumor potential.



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1. Introduction

Cancer development is a multifactorial process, resulting from mutations of cells that tend to increase in size, as they lack ions, glucose and nutrients, in addition to having inadequate oxygenation mediated by HIF (Wu et al. 2018). HIF is a heterodimer complex, consisting of two subunits: HIF-1α and HIF-1β, the former being constitutive and extremely sensitive to changes in oxygen levels (Bandarra and Rocha 2013). On the other hand, NF-κB is a family of transcription factors that regulate immune, inflammatory and oncogenic responses (Hoesel and Schmid 2013). Finally, the NF-κB and HIF-1α transcription factors are intimately connected by sharing many stimuli capable of activating and attacking genes, so they both cooperate to create the necessary conditions for the tumour to enlarge and become aggressive (Bandarra and Rocha 2013).

Jatropha (Euphorbiaceae) is a genus of approximately 199 species. Its extracts are obtained from different parts such as leaves, stem, bark and roots, and have been used in the ethnomedicine of different cultures (Duke 1985). Several compounds with antitumor activity have been isolated from these species, e.g., *J. isabellei* (Fröhlich et al. 2013); *J. podagrica* (Ghali et al. 2013); *J. multifida* (Das et al. 2009); *J. neopauciflora* (García and Delgado 2006) and *J. curcas* (Juan et al. 2003). *Jatropha macrantha* Müll. Arg. also known as 'Huanarpo Macho', is employed in the traditional medicine of the Peruvian region as a blood depurative and antidiabetic, in the treatment of respiratory diseases and as a stimulator of the nervous system (Condori et al. 2018). It is also used in poultice form in traditional medicine, for the treatment of skin ulcers (Sabandar et al. 2013).

Our study started by accounting for how other plants of this genus have shown promising potential for anti-tumour compounds, e.g., curcin isolated from *J. curcas* showed an IC_{50} of 0.19 nmol/l in the SGC-790 tumour cell line; diterpenoids curcusone C, curcusone D, multidione, 15-epi-4Z-jatrogrossidentadion, 4E-jatrossidentadion, 4Z-jatrossidentadion, 2-hydroxyjatrogrossidion and 2-epi-hydroxyisojatrogrossidion isolated from *J. curcas* showed a strong action against the HeLa tumour cell line; calenduladiol and (3β,16β)-16-hydroxylup-20-(29)-en-3-yl(E)-3-(4-hydroxyphenyl) prop-2-enoate isolated from *J. neopauciflora* exhibited activity potential against the K562 and U251 tumour cell lines (Cavalcante et al. 2020). Thus, our study aims to identify and characterise the compound(s) responsible for inhibiting the production of HIF-1α and NF-κB in extracts of *J. macrantha* by chromatographic methods and spectrophotometric analysis.

2. Results and discussion

Pomolic acid (**1**) and euscaphic acid (**2**) (Figure 1) were isolated from the CH_2Cl_2 extract prepared from *J. macrantha* which was repeatedly subjected to several column chromatography. Both compounds are reported for the first time in the *J. macrantha* species.

The extracts obtained from *J. macrantha* did not show cytotoxic effects in hypoxic conditions at 72 h of treatment in the SK-MEL-28, A549 and U-373 MG cells by means of the XTT assay. In addition, the pomolic acid (**1**) and euscaphic acid (**2**) compounds

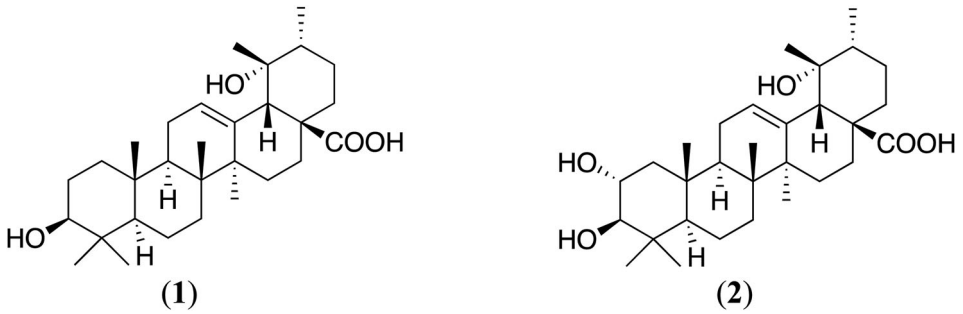

Figure 1. Absolute configuration of pomolic acid (**1**) and euscaphic acid (**2**).

showed a cytotoxic effect against the SK-MEL-28, A549 and U-373 MG cancer cell lines (Supplementry material, Table S1).

The inhibitory activity of the extracts and isolated compounds over NF-κB under hypoxic conditions on different cancer cell lines at 72 h of treatment is reported in Supplementry material, Table S2, and their effect was compared with that of the positive control, JSH-23 (IC_{50}=7.1 μM). The CH_2Cl_2 extract revealed a greater inhibitory effect on NF-κB compared to the *n*-hexane, MeOH and aqueous extracts.

Pomolic acid (**1**) inhibited the production of NF-κB on the SK-MEL-28, A549 and U-373 MG cancer cell lines with IC_{50} of 1.05 ± 0.02 μM, 3.63 ± 0.01 μM and 2.55 ± 0.02 μM. Our data corroborates the antitumor activity of pomolic acid described by other authors on breast cancer cell lines (MCF7, MDA-MB-231) with an IC_{50} of 25 μM and 10 μM, respectively (Kim et al. 2016). Euscaphic acid (**2**) inhibited the production of NF-κB with IC_{50} of 2.71 ± 0.01 μM, 3.73 ± 0.02 μM and 3.39 ± 0.01 μM on SK-MEL-28, A549 and U-373 MG cancer cells, respectively. The results show that euscaphic acid has NF-κB dose-dependent inhibition activity in all the tested tumoral cell lines, inhibiting IKKα/β phosphorylation and IκBα phosphorylation, which subsequently causes the blockage of NF-κB p65 phosphorylation and nuclear translocation (Kim et al. 2012; Ouyang et al. 2019). Likewise, Dai et al. (2019), reported that euscaphic acid reduced cell proliferation and induced apoptosis and cell cycle arrest in Nasopharyngeal carcinoma cells by suppressing the PI3K/AKT/mTOR signalling pathway.

Analysing both mechanisms, we can indicate that there is a relationship between the NF-κB and PI3K/Akt/mTOR signalling pathways. In this sense, mTOR is known to regulate Akt via a feedback mechanism. The regulation of NF-κB by mTOR happens through Akt regulation and by means of the mTOR-associated protein raptor. Likewise, this can occur due to a more direct regulation of NF-κB by mTOR which involves IKK. In the activation of NF-κB, IKK plays an important role by phosphorylating the inhibition of IκBα leading to its dissociation from NF-κB, and resulting in an activated NF-κB that translocate to the nucleus. Thus, the mTOR mediated induction of IKK leads to the activation of NF-κB (Ahmad et al. 2013).

The inhibitory activity of the extracts and compounds over HIF-1α under hypoxic conditions on different cancer cell lines at 72 h of treatment is reported in Supplementry material, Table S3 and compared to the positive control, 2-MeOE2 (IC_{50}=0.5 μM). The CH_2Cl_2 extract revealed a greater inhibitory effect on HIF-1α compared to n-hexane, MeOH and aqueous extracts.

Pomolic acid (**1**) inhibited HIF-1α with IC_{50} of $3.01 \pm 0.02\ \mu M$, $9.97 \pm 0.01\ \mu M$ and $6.34 \pm 0.02\ \mu M$ on the SK-MEL-28, A549 and U-373 MG cells, respectively. We can compare these results to those obtained by Park *et al*. reporting an IC_{50} of $10\ \mu M$ on the control cell lines (HUVEC) and the MCF-7 and MDA-MB-231 tumoral cell lines in normoxic conditions (Park et al. 2016). Euscaphic acid (**2**) inhibited HIF-1α with IC_{50} of $3.78 \pm 0.02\ \mu M$, $10.25 \pm 0.01\ \mu M$ and $8.85 \pm 0.02\ \mu M$ on the SK-MEL-28, A549 and U-373 MG cells, respectively.

This is the first report on the inhibitory activity of euscaphic acid against the HIF-1α transcription factor. Previous research showcased triterpenoids preventing HIF-1α translocation in the nucleus, where they are heterodimised with HIF-1β to form HIF. This together with the p300/CBP co-activators form a transcription complex that binds to target genes and activates the cascade of pro-angiogenic, prooncogenic and inflammatory events (Bahadori et al. 2019). Since the isolated compounds are also terpenoids, we can reasonably assume that their mechanism of action is like that described by Bahadori *et al*.

Our results regarding the response of extracts and compounds (pomolic and euscaphic acids) isolated of *J. macrantha* on the HIF-1α and NF-κB targets confirm that there are positive correlations between these targets and that both are involved in cancer progression and tumour responses in hypoxic conditions. Since the HIF-1α and NF-κB regulatory pathways are a highly complex network involving several signalling cascades and overlapping mechanisms, each one of them could serve as a promising target or step to intervene tumours in the future.

Author contributions

LAT and ARS contributed to the analysis of the spectral data; LAT and GP contributed to the conception and experimental design of the pharmacological study and LAT and FA contributed to the writing and review of the manuscript.

Disclosure statement

The authors declare no conflict of interest.

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Triterpenoids isolated from *Jatropha macrantha* (Müll. Arg.) inhibit the NF-κB and HIF-1α pathways in tumour cells

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ABSTRACT

Activity-guided fractionations of *Jatropha macrantha* Müll. Arg. led to the isolation of pomolic acid (**1**) and euscaphic acid (**2**). The potential for inhibition against NF-κB and HIF-1α production of these two compounds was tested in different tumour cell lines. Compounds **1** and **2** showed an inhibitory activity of HIF-1α in the SK-MEL-28 (IC_{50}=3.01±0.02 μM and 3.78±0.02 μM), A549 (IC_{50}=9.97±0.01 μM and 10.25±0.01 μM) and U-373 MG (IC_{50}=6.34±0.02 μM and 8.85±0.02 μM) cell lines. In addition, compounds **1** and **2** showed an inhibitory activity on NF-κB in SK-MEL-28 (IC_{50}=1.05±0.02 μM and 2.71±0.01 μM), A549 (IC_{50}=3.63±0.01 μM and 3.73±0.02 μM) and U-373 MG (IC_{50}=2.55±0.02 μM and 3.39±0.01 μM) cell lines. This is the first report that isolates these compounds from *J. macrantha* and tests their antitumor potential.

Keywords:

Euphorbiaceae, *Jatropha macrantha*, NF-κB, HIF-1α, Sterols, Triterpenoids.

Abbreviations

2-MeOE2	2-Methoxyestradiol
ATCC	American Type Culture Collection
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle Medium
EPO	Erythropoietin
FBS	Fetal Bovine Serum
HRE	Hypoxia Response Element
HIF	Hypoxia-inducible factor
JSH-23	4-methyl-1-N-(3-phenylpropyl)-1,2-benzenediamine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBMCs	Peripheral blood mononuclear cell
PMS	Phenazine methosulfate
PBS	Phosphate-Buffered Saline
RLU	Relative light units
RPMI	Roswell Park Memorial Institute
TNF-α	Tumour necrosis factor-α

Experimental

Plant material

Jatropha macrantha was collected from in Paucarpata municipality, in the Arequipa province of Arequipa department, Peru (16°25'46" S 71°30'08" W), in September 2015, at an altitude of 2410 m. Botanical identification was confirmed by the National Herbal of Medicinal Plants, National Institute of Health, CENSI, and a voucher specimen was deposited (HINS 02334).

General experimental procedures

First grade organic solvents were used for isolating the compounds and they were purchased from Sigma-Aldrich. TLC was performed using Merck Silica gel 60-F_{254} plates. Chromatograms thus obtained were visualised by UV absorbance (254 nm) and through heating a plate stained with phosphomolybdic acid. Column chromatography was performed with silica gel (20-45 μm and 40-63 μm, Merck). NMR experiments were performed on the Bruker Advance DRX 300, 500 and 600 spectrometers operating at 300 MHz, 500 MHz, 600 MHz (^{1}H) or 126 MHz, and 151 MHz (^{13}C). The deuterated solvents were $CDCl_3$-d_1, MeOD-

d_4 and DMSO-d_6. Spectrums were calibrated by assignment of the residual solvent peak to δ_H 7.26, δ_H 3.31 and δ_H 2.5, and δ_C 77.16, δ_C 49.00 and δ_C 39.52, for $CDCl_3$, MeOD and DMSO, respectively. The complete assignment of protons and carbons was done by analysing the correlated ^{1}H-^{1}H COSY, HSQC and HMBC spectrums. Mass spectrums were recorded by the VG AutoSpec equipment from Waters, with magnetic sector analyser. The FAB ionisation technique used L-SIMS with cesium cation bombardment as an energy source, and the *m*-nitrobenzyl alcohol as a matrix.

Preparation of extracts and isolation of compounds

After air-drying and powdering *J. macrantha* (200 g), the result was repeatedly extracted at room temperature with *n*-hexane (13.60 g), CH_2Cl_2 (2.36 g) and MeOH (44.36 g), as well as from an aqueous extract (30.70 g). Each extract was evaluated for its cytotoxic effects and HIF-1α/NF-κB production on the SK-MEL-28, A549 and U-373 MG cells and the nontumorigenic PBMCs cell line. All extracts did show viability in the non-cancerous cell line (PBMCs). With respect to the activity assays, the dichloromethane and *n*-hexane extracts of *J. macrantha* showed a reduction in the production of HIF-1α/NF-κB. However, the methanol and aqueous extracts did not show activity in terms of reduction in the production of HIF-1α/NF-κB. Therefore, the dichloromethane extract was fractionated to identify the active compounds.

The bioactive CH_2Cl_2 extract (2 g) was analysed through bioassay-guided Silica gel (40-63 μm) column chromatography (2x50 cm), using a step-wise gradient of *n*-hexane/ CH_2Cl_2 (1:1 to 3:0.5 v:v), obtaining four fractions (1-4), where **fractions 1** (43.7 mg) and **4** (21 mg) showed greater activity (inhibition of the activity of HIF-1α and NF-κB). **Fraction 1** was a pure compound–**compound 1**, while **fraction 4** needed a new separation through a Silica gel column chromatography (2x50 cm) (20-45 μm) (*n*-hexane/ethyl acetate, 6:0.5), obtaining five fractions (4A-4E) that showed inhibition over HIF-1α and NF-κB, with the **fraction 4D**–**compound 2** (3.1 mg) being the most active.

Cell culture

Three human cancer cell lines were used in this study: Human skin melanoma (SK-MEL-28, ATCC® HTB-72), Human lung carcinoma (A549, ATCC® CRM-CCL-185™), Human brain astrocytoma (U-373 MG, ATCC® HTB-17) cancer cell lines were selected to carry out the assays on the extracts and compounds from *Jatropha macrantha*. We have selected these cancer cell lines according to their physiological link with particularly relevant diseases against which *Jatropha macrantha* is used in traditional medicine (Condori *et al*., 2018;

Pardo, 2002; Desmarchelier *et al*., 1996). In this sense, the SK-MEL-28 cell lines were selected due to *J. macrantha* traditional use in the treatment of skin ulcers, the A549 cell line was selected because of *J. macrantha* use as a cough suppressant and anti-asthmatic, and the U-373 MG cell line was selected in relation to *J. macrantha* use as a cerebral tonic. In addition, PMBCs was used to assess the safety of the compounds. PBMCs from healthy donors were isolated from the blood by Ficoll (Rafer) density gradient centrifugation in Leucosep tubes (Greiner Bio-One) following the manufacturer's recommendations. All experiments with human PBMC were carried out in accordance with the Declaration of Helsinki, and all investigations were approved by the ethics committee of the Hospital Universitario La Paz (No. 913/2018). All cell lines were obtained from the ATCC. Cells were cultured in specific media according to ATCC recommendations. PMBCs were obtained from whole blood by density gradient centrifugation using Lymphoprep (StemCell Technologies), according to the manufacturer's instructions. In both cases the incubation condition was established at 37°C in hypoxic conditions (1% O_2), thus mimicking the *in vivo* tumour microenvironment.

Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, USA) supplemented with L-glutamine (PanReac AppliChem, Barcelona, Spain), 10% FBS (Summit Biotechnology; Ft. Collins, CO), 100 U/mL penicillin and 100 μg/mL streptomycin (Fisher Scientific, Pittsburgh, USA). Test compounds were dissolved in DMSO (Merck) at a 10 mM concentration, while the extracts and fractions were dissolved at 20 mg/mL in DMSO.

Viability assay

Inhibition of H_2O_2-induced viability by the extracts at various concentrations was tested by the method of XTT-formazan dye formation (Weislow *et al*., 1989), using the above mentioned cell lines. These cells were sown (200 μL, $2x10^4$ cells/well) in a 96-well plate and allowed to grow at 37ºC. After 72 h, medium was removed from all wells. 200 μL fresh medium (DMEM medium supplemented with L-glutamine, 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin) was added to the control wells. Cells in each test well were treated with 0.1 mM H_2O_2 (prepared in medium) along with extracts or compounds in different concentrations (100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.12 μM, 1.56 μM, 0.78 μM, 0.39 μM, 0.95 μM). Concerning viability, the results were compared with those of Docetaxel as positive control (IC_{50}=5.7 *n*M). For this assay, Docetaxel was chosen. It is a chemotherapy medication used to treat various types of cancer. The concentration used in the assay was based on previous work (Florento *et al.*, 2012). Cells in both control and test wells were re-incubated for 8 h maintaining the same conditions. After the treatment incubation

period, medium in each well was substituted by 200 μL of fresh medium, followed by the addition of 50 μL of XTT (0.6 mg/ml) containing 25 μM PMS. The plate was further incubated for 4 h in the same conditions. Absorbance was measured at 450 nm (with a 630 nm reference filter) in a spectrophotometric ELISA plate reader (SpectraMax® i3, Molecular Devices, CA, USA).

NF-κB inhibition assay

Cells were transfected using a lipofectamine plus transfection reagent (Thermo Fisher Scientific) with 0.3 μg of the NF-κB-promoted luciferase reporter gene plasmid (pGL2-NF-κB-Luc) (Promega), and 0.03 μg of the Renilla luciferase reporter plasmid (pTK-Renilla) (transfection normalisation vector, Promega). After 1 day, the cells were incubated with TNF-α (5 *n*g/mL) in the absence or presence of the extracts or the isolated compounds at different concentrations (100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.12 μM, 1.56 μM, 0.78 μM, 0.39 μM, 0.95 μM) for 72 h of treatment in hypoxic conditions (1% O_2). With respect to the inhibition of NF-κB, the results were compared to that of JSH-23, used as positive control (IC_{50}=7.1 μM). It is an inhibitor of NF-kB nuclear translocation. The concentration used in the assay was based on previous work (Kumar *et al.*, 2011). Subsequently, the cells were lysed in 25 mM Tris-phosphate, at pH 7.8, containing 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and 7% glycerol. The luciferase activity was measured by Dual-Luciferase Reporter Assay Kit (Promega), according to the manufacturer's instructions. Luminescence was measured immediately in triplicates using a GloMax 96 Microplate Luminometer (Promega). The experiments for each concentration of the samples were performed using 3 replicates of each well in three different experiments.

HIF-1α inhibition assay

Cells have been stably transfected with EPO-Luc plasmid. EPO-HRE-luciferase plasmid reporter contained three copies of the HRE consensus sequence from the promoter of the erythropoietin gene in the pGL3 vector. Cells ($1x10^4$) were seeded the day before the assay. The next day, the cells were stimulated with the extracts and the isolated compounds at different concentrations (100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.12 μM, 1.56 μM, 0.78 μM, 0.39 μM, 0.95 μM). With respect to the inhibition of HIF-1α, results were compared with those of the positive control, 2-MeOE2 (IC_{50}=0.5 μM), an inhibitor of angiogenesis, microtubule disruption and upregulation of the extrinsic and intrinsic apoptotic pathway. The concentration used in the assay was based on previous work (Zhao *et al.*, 2017). After 72 h of stimulation in hypoxic conditions (1% O_2), the cells were lysed in 25

mM Tris-phosphate, at pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and 7% glycerol during 15 min at room temperature in a horizontal shaker. The luciferase activity was measured using a GloMax 96 microplate luminometer (Promega) following the instructions of the luciferase assay kit (Promega). The RLU (Relative light units) was then calculated and the results were expressed as percentage of inhibition induction/inhibition of EPO-luc activity. Luminescence was measured immediately in triplicates using a GloMax 96 Microplate Luminometer (Promega). The experiments for each concentration of the samples were performed using 3 replicates of each well in three different experiments.

Statistical analysis

The statistical significance of differences was calculated employing the GraphPad Prism software, version 8.2.1 (GraphPad Software Inc., San Diego, CA, USA), using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Results were considered significant when $p<0.01$. IC_{50} values were determined by non-linear regression using GraphPad Prism, version 8.2.1. All the experiments were performed in triplicate.

Spectroscopic data

Pomolic acid (**1**): Colourless crystalline powder; m.p.=299ºC; $[\alpha]^{20}_D$ +36.7 (*c* 2.0, THF); ^{1}H-NMR (500 MHz, DMSO) δ_H=5.13 (1H, s, H-12), 2.99 (1H, m, H-3), 2.43 (1H, m, H-16β), 2.37 (1H, m, H-18), 1.88 (1H, m, H-11β), 1.83 (1H, m, H-11α), 1.69 (1H, m, H-15α), 1.68 (1H, m, H-2β), 1.60 (1H, m, H-21α), 1.58 (1H, m, H-22β), 1.56 (1H, m, H-9), 1.52 (1H, m, H-1β), 1.50 (1H, m, H-22α), 1.49 (1H, m, H-1α), 1.47 (1H, m, H-6β), 1.45 (1H, m, H-7β), 1.38 (1H, m, H-16α), 1.31 (1H, m, H-6α), 1.28 (3H, s, Me-27), 1.26 (1H, m, H-20), 1.22 (1H, m, H-7α), 1.12 (1H, m, H-21β), 1.06 (3H, s, Me-29), 0.93 (3H, s, Me-23), 0.90 (1H, m, H-15β), 0.88 (3H, s, Me-25), 0.83 (3H, s, Me-30), 0.71 (3H, s, Me-26), 0.69 (3H, s, Me-24), 0.67 (1H, m, H-5); ^{13}C-NMR (126 MHz, DMSO) δ_C=179.55 (C-28), 138.91 (C-13), 126.85 (C-12), 71.85 (C-19), 77.15 (C-3), 55.05 (C-5), 53.44 (C-18), 47.10 (C-17), 46.90 (C-9), 41.59 (C-20), 41.24 (C-14), 38.54 (C-4), 38.33 (C-10), 37.50 (C-1), 36.72 (C-22), 32.88 (C-7), 28.40 (C-23), 28.30 (C-2), 27.09 (C-15), 26.61 (C-29), 26.15 (C-21), 25.43 (C-16), 24. 09 (C-27), 23.30 (C-11), 18.28 (C-6), 16.83 (C-26), 16.48 (C-30), 16.18 (C-24), 15.25 (C-25); HRESIMS m/z 472.3539 $[M+H]^+$ (calcd for $C_{30}H_{48}O_4$). Data was compared to the reference from Zhao *et al*., 2019.

Euscaphic acid (**2**): Colourless crystalline powder; m.p.=273ºC; $[\alpha]^{23}_D$ +12.2 (*c* 1.0, MeOH); ^{1}H-NMR (600 MHz, DMSO) δ_H=5.17 (1H, brs, H-12), 3.75 (1H, dt, H-2β), 3.15

(1H, d, H-3β), 2.47 (1H, ddd, H-16), 2.37 (1H, s, H-18), 1.29 (3H, s, Me-27), 1.08 (3H, s, Me-29), 0.88 (3H, s, Me-23), 0.88 (3H, s, Me-25), 0.84 (3H, d, Me-30), 0.78 (3H, s, Me-26), 0.69 (3H, s, Me-24); ^{13}C-NMR (151 MHz, DMSO) δ_C=179.01 (C-28), 138.70 (C-13), 126.79 (C-12), 77.94 (C-3), 71.67 (C-19), 64.72 (C-2), 53.21 (C-18), 47.68 (C-5), 46.91 (C-17), 46.57 (C-9), 41.61 (C-20), 41.43 (C-14), 41.20 (C-1), 38.01 (C-8), 37.83 (C-10), 37.31 (C-4), 32.66 (C-22), 28.91 (C-7), 28.03 (C-15), 26.45 (C-23), 25.97 (C-16), 25.20 (C-29), 24.10 (C-21), 23.19 (C-27), 21.87 (C-11), 17.75 (C-6), 16.68 (C-26), 16.32 (C-24), 16.32 (C-25), 16.14 (C-30); HRESIMS m/z 488.3428 $[M+H]^+$ (calcd for $C_{30}H_{48}O_5$). Data was compared to the reference from Woo *et al*., 2014.

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- Figure S23. HMBC spectrum of JMEDCM_F4D in DMSO 600 MHz
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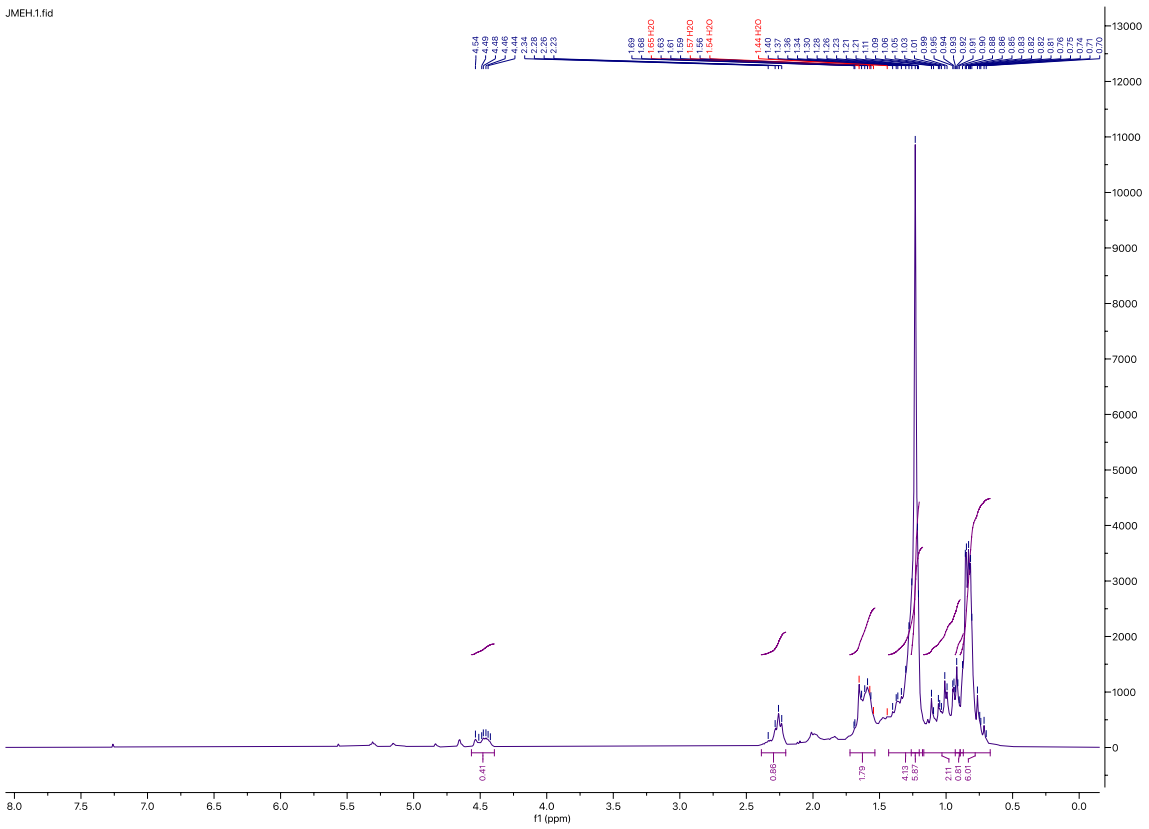
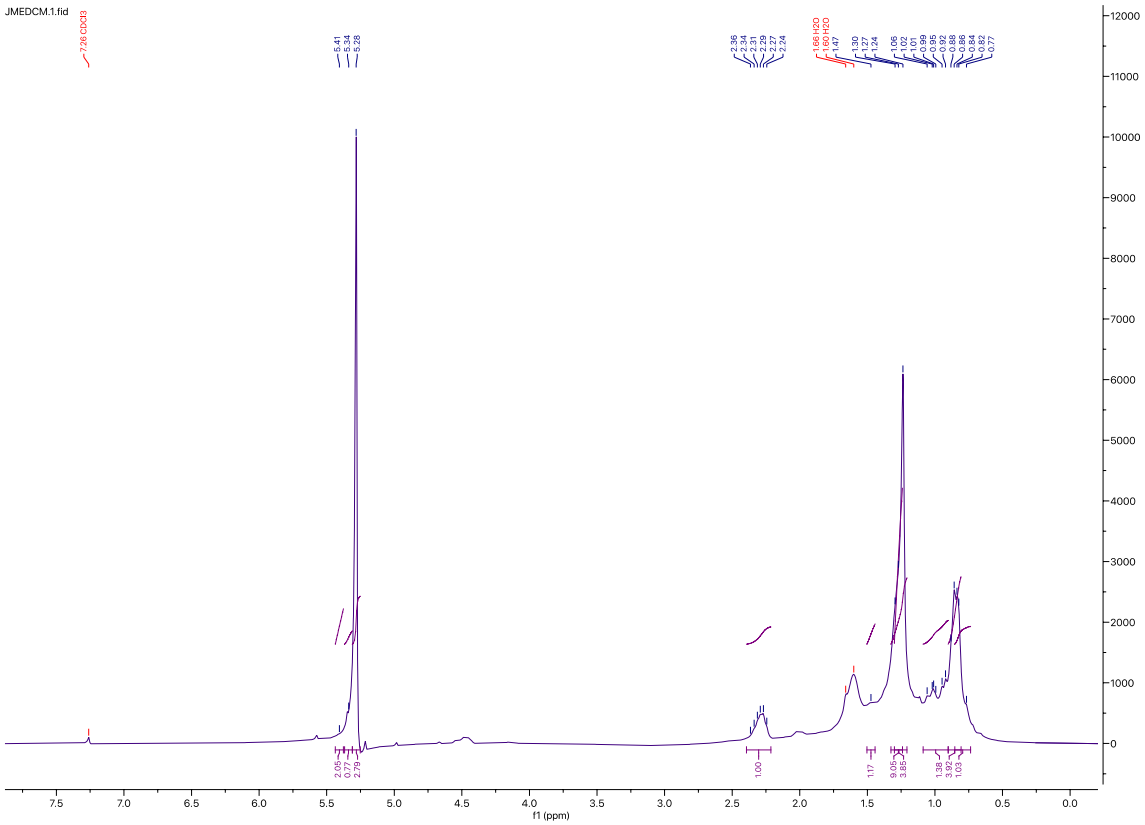
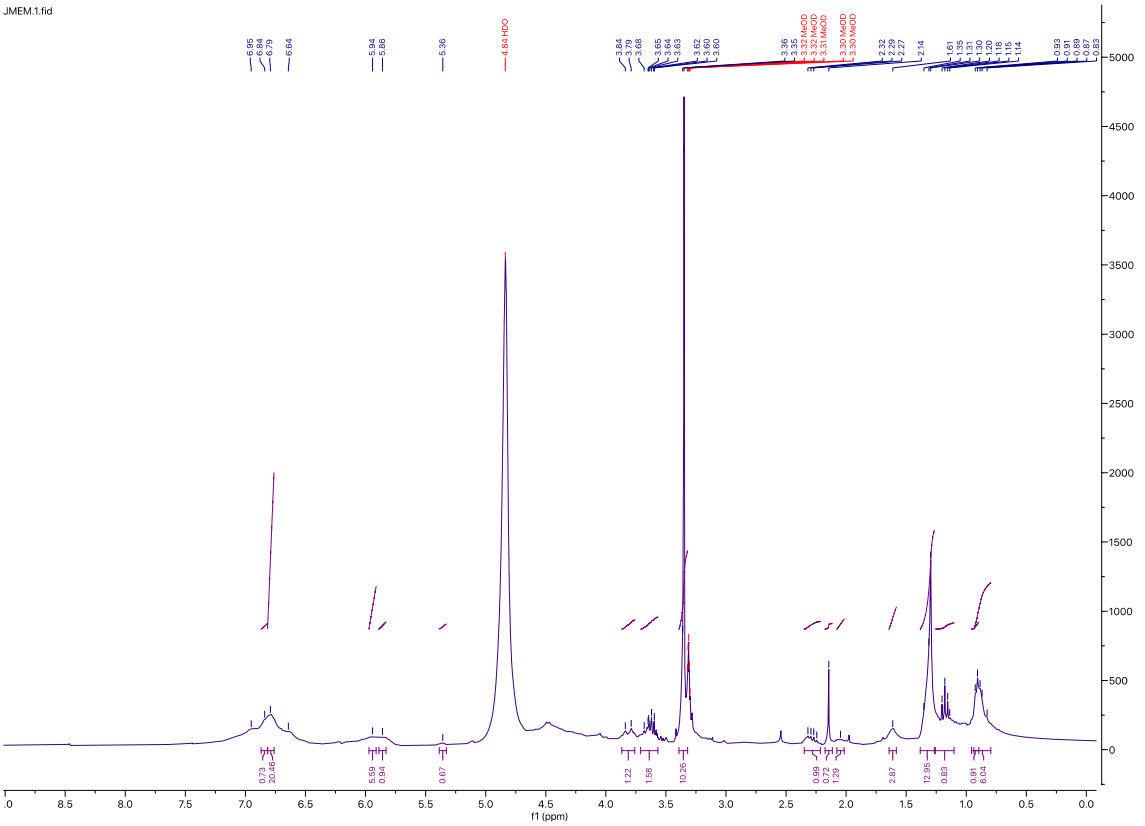
- Figure S26. HRMS spectrum of JMEDCM_F4D
- Table S1. XTT viability assay of *J. macrantha* extracts and compounds against a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic (1% O_2) conditions. Data presents the means (±SD) of three separate experiments performed in triplicate. Docetaxel was used as positive control.
- Table S2. Inhibitory effect of *J. macrantha* extracts and compounds on NF-κB activation in a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic (1% O_2) conditions. The results are the means (±SD) of three separate experiments performed in triplicate. JSH-23 was used as positive control.
- Table S3. Inhibitory effect of *J. macrantha* extracts and compounds on HIF-1α in a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic conditions. The results are the means (±SD) of three separate experiments performed in triplicate. 2-MeOE2 was used as positive control.



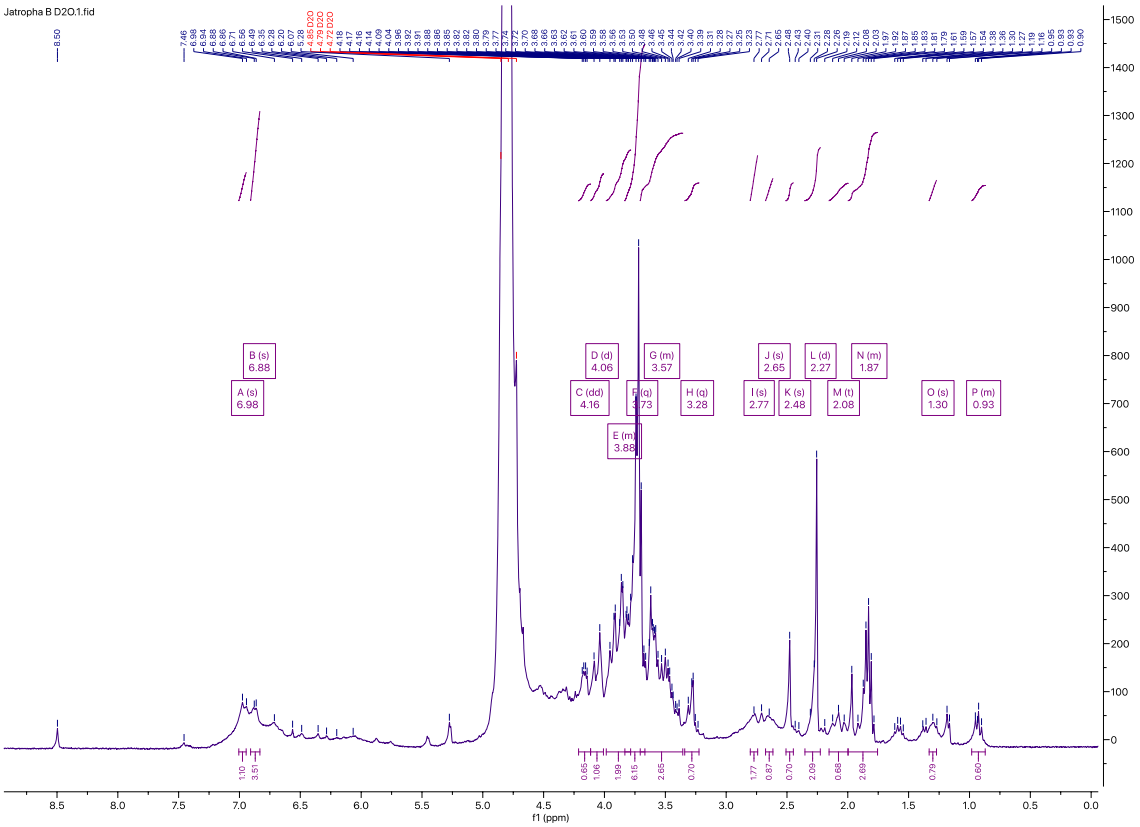
-Figure S1. ^{1}H-NMR spectrum of n-hexane extract of *J. macrantha* in $CDCl_3$ 300 MHz



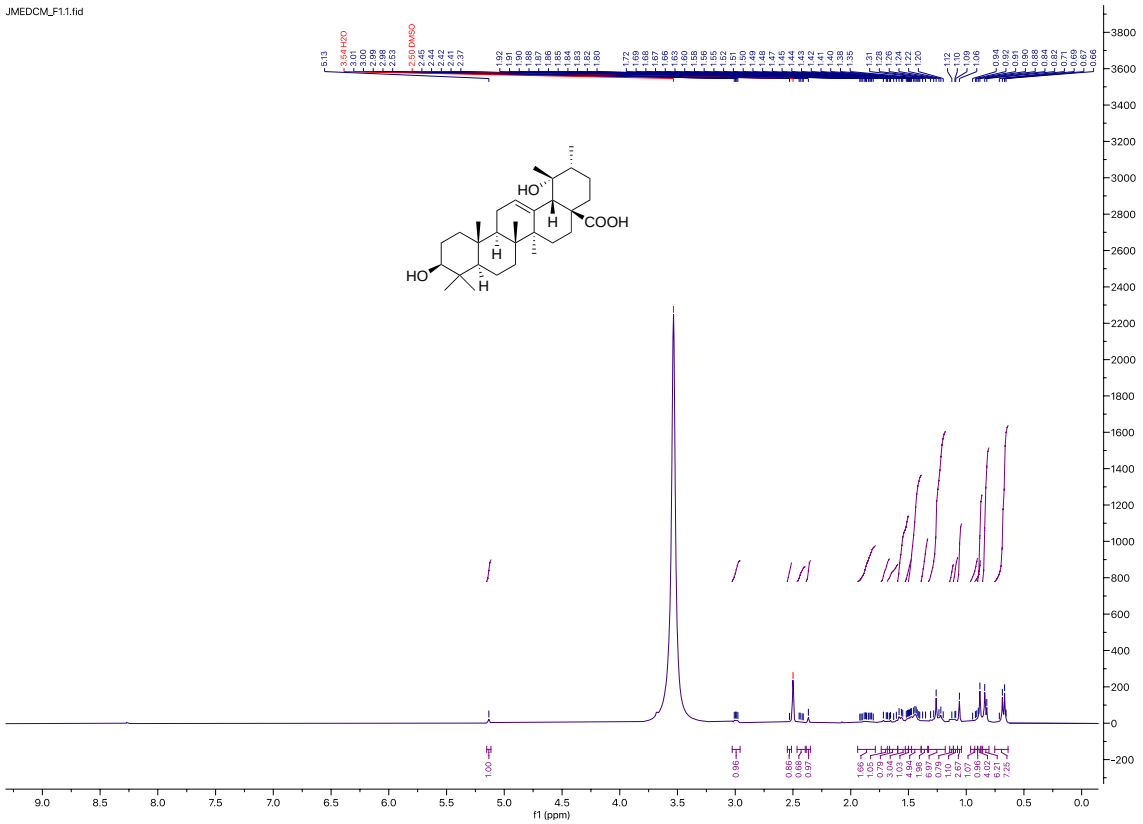
-Figure S2. ^{1}H-NMR spectrum of dichloromethane extract of *J. macrantha* in $CDCl_3$ 300 MHz



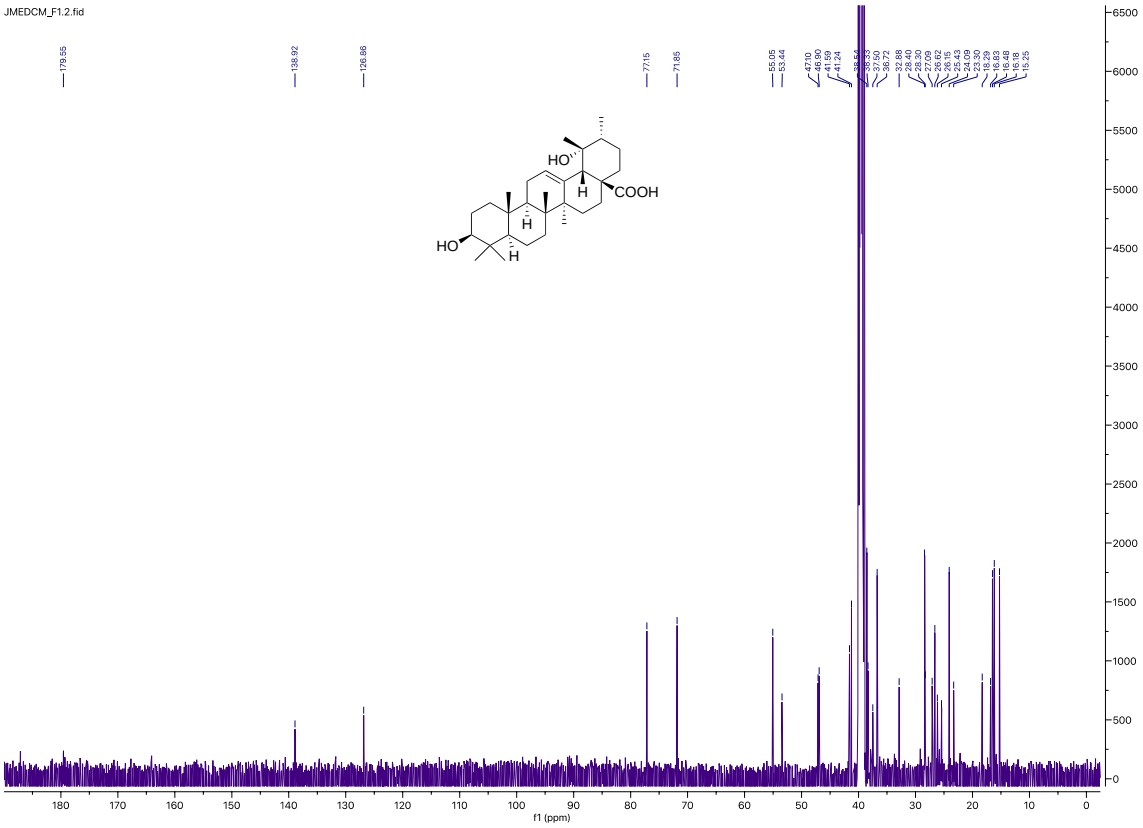
-Figure S3. ^{1}H-NMR spectrum of methanol extract of *J. macrantha* in MeOD 300 MHz



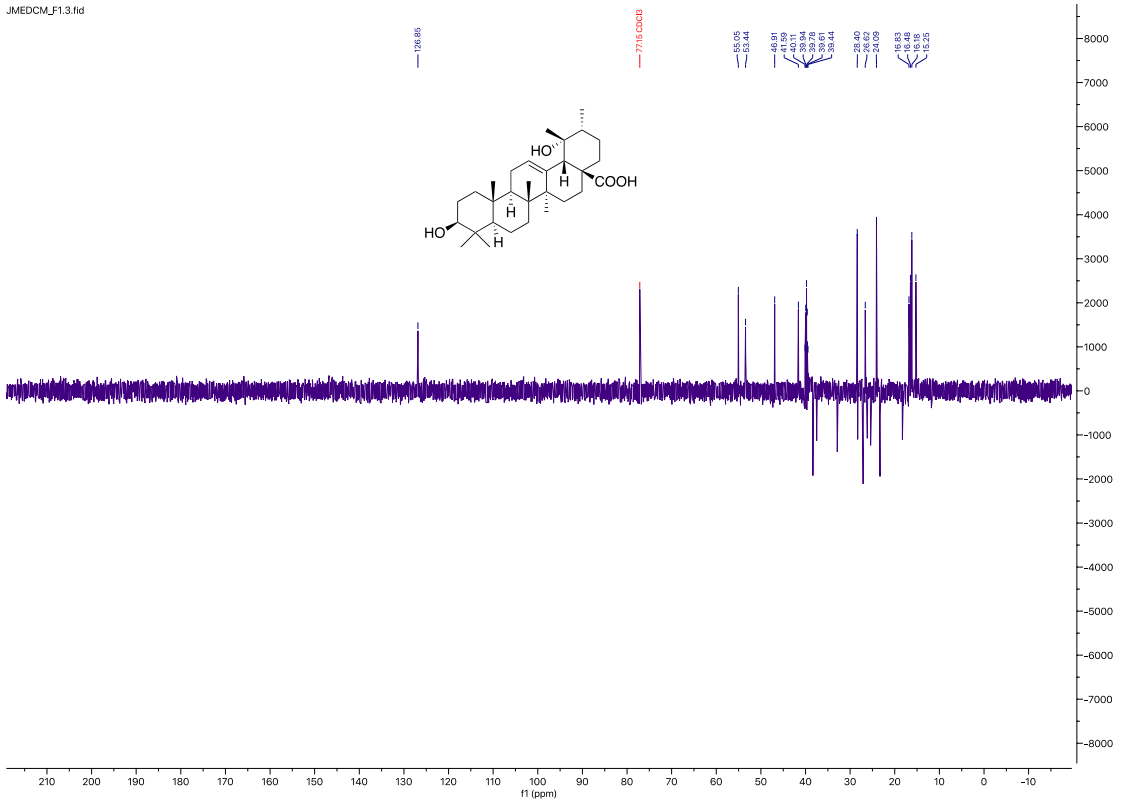
-Figure S4. ^{1}H-NMR spectrum of aqueous extract of *J. macrantha* in D_2O 300 MHz



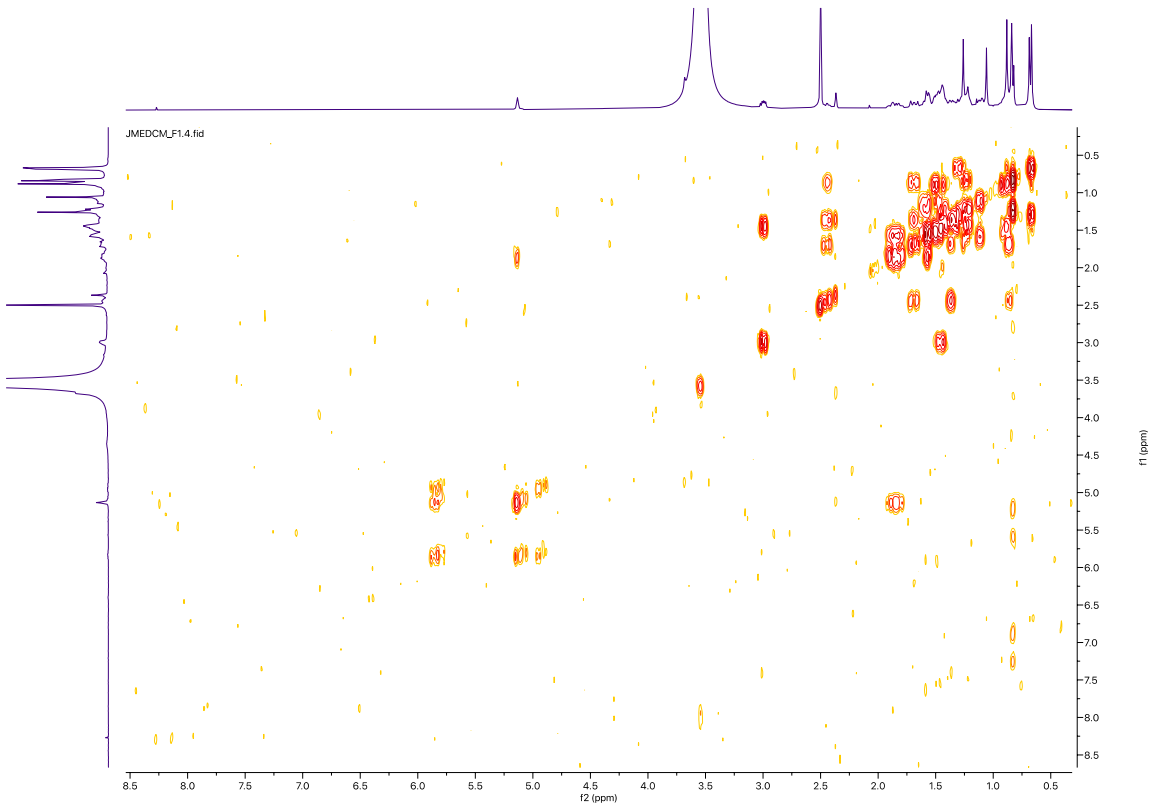
-Figure S5. ^{1}H-NMR spectrum of JMEDCM_F1 in DMSO 500 MHz



-Figure S6. ^{13}C-NMR spectrum of JMEDCM_F1 in DMSO 126 MHz.

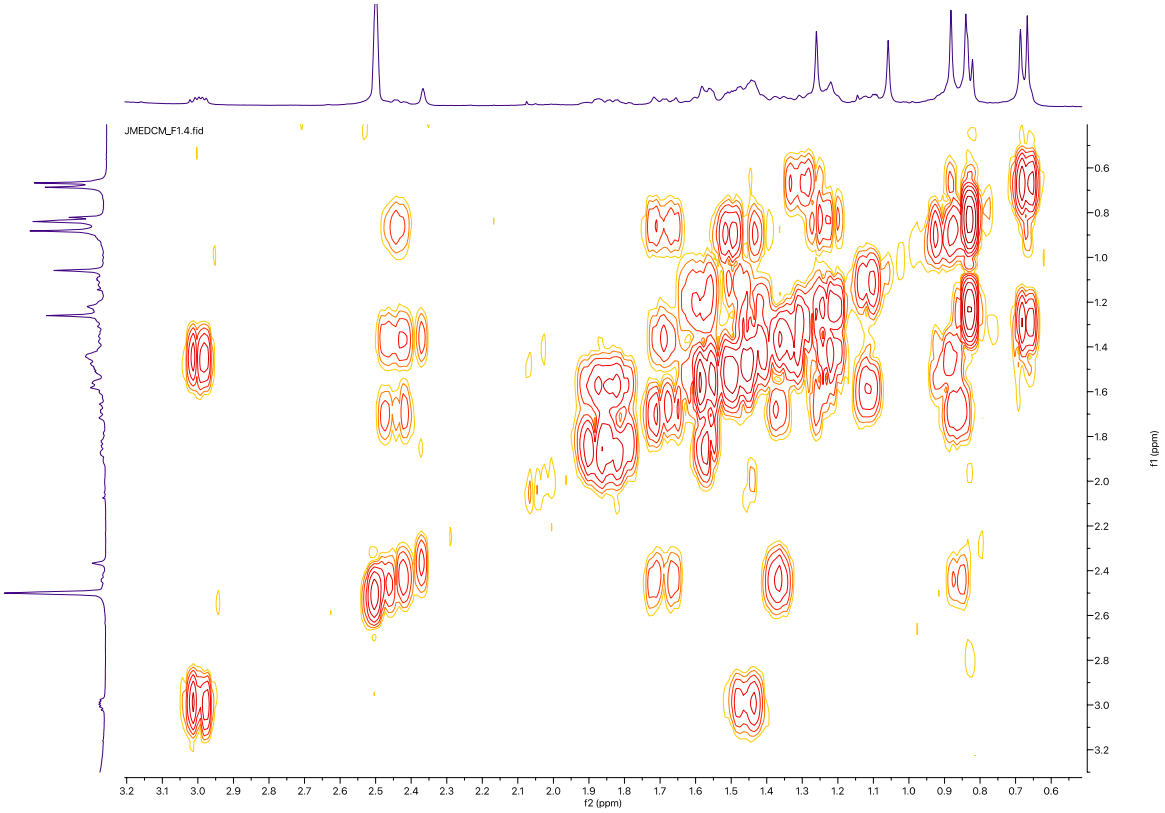


-Figure S7. DEPT-135 spectrum of JMEDCM_F1 in DMSO 126 MHz.

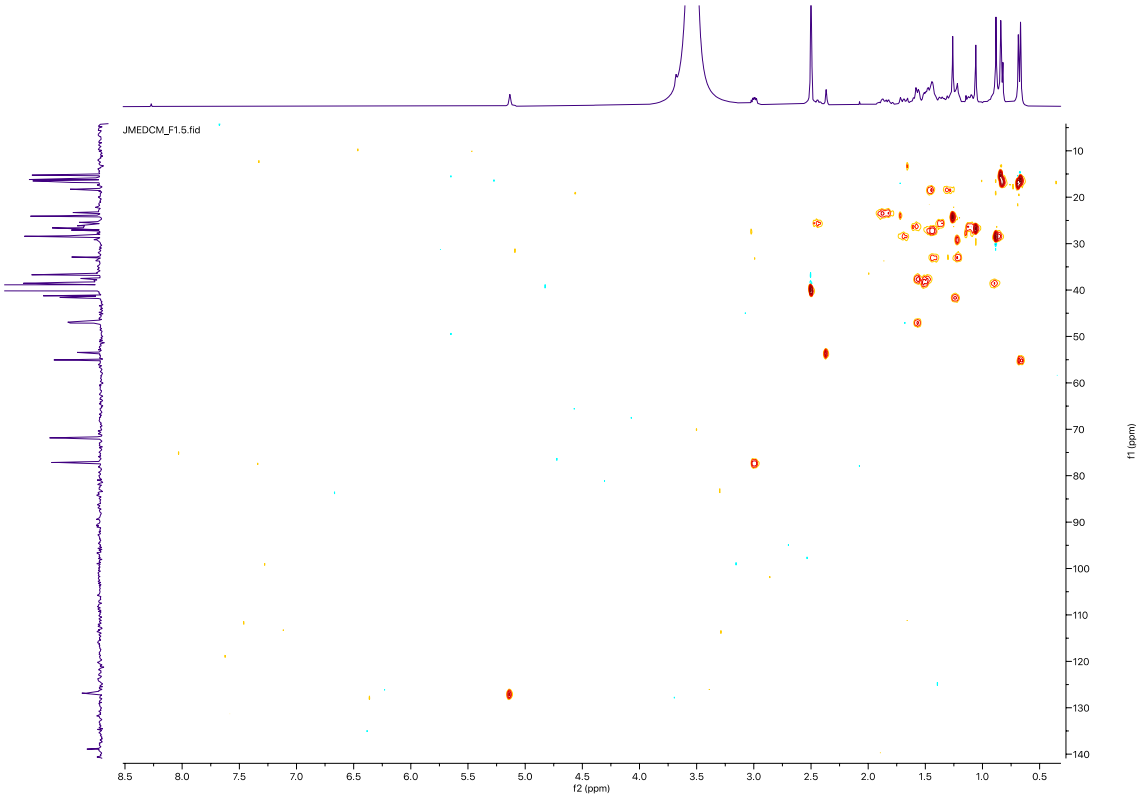


-Figure S8. ^{1}H-^{1}H COSY spectrum of JMEDCM_F1 in DMSO 500 MHz.

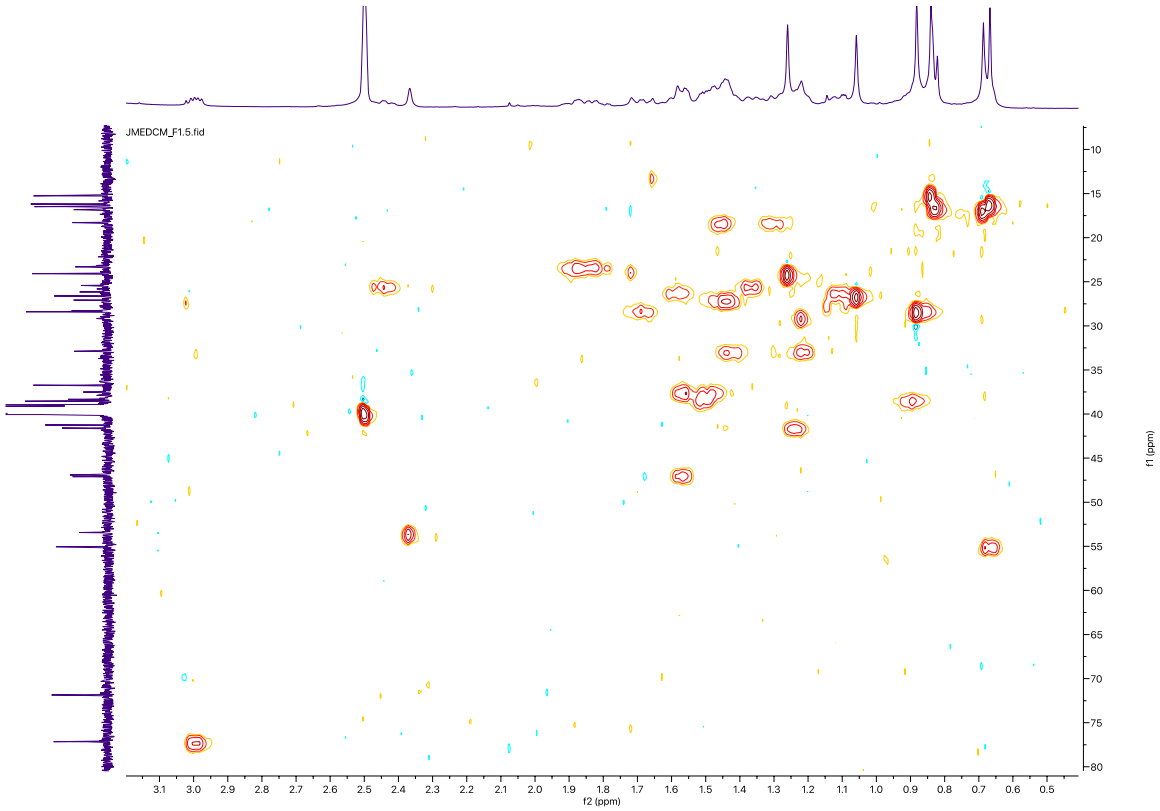
-Figure S9. ^{1}H-^{1}H COSY spectrum of JMEDCM_F1 in DMSO 500 MHz (Extension 1).



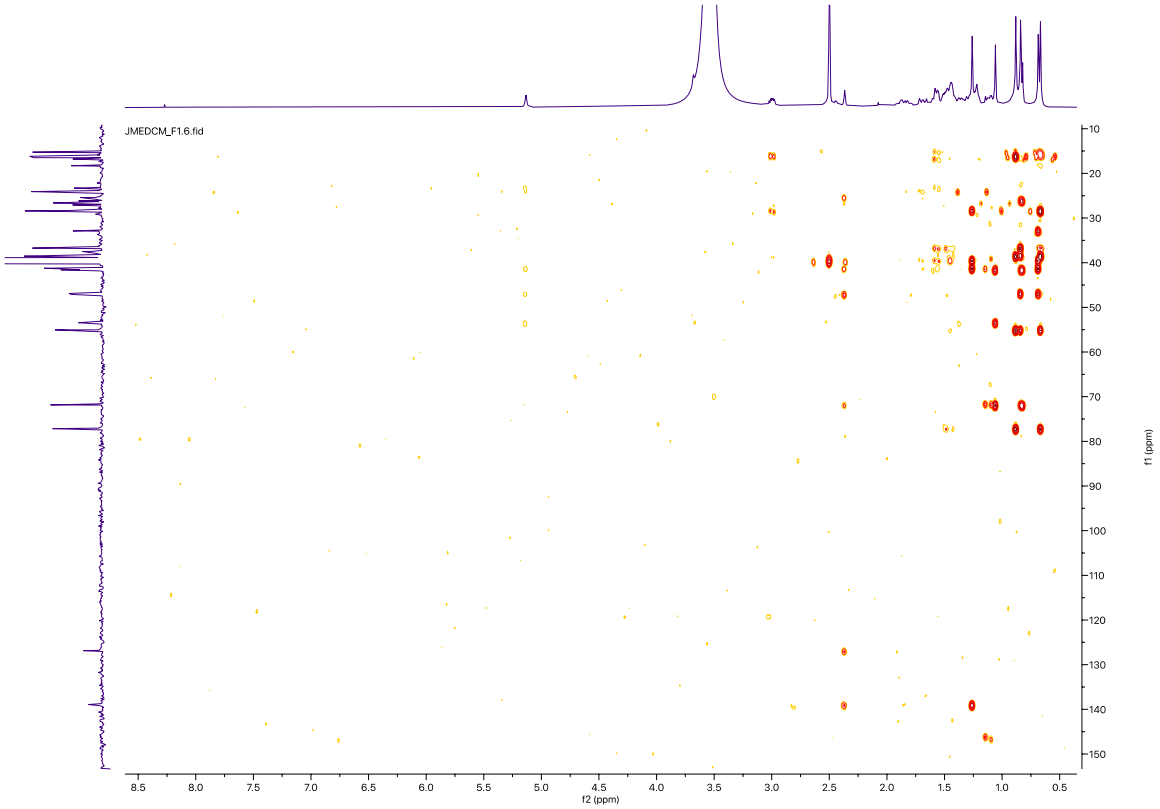
-Figure S10. ^{1}H-^{1}H COSY spectrum of JMEDCM_F1 in DMSO 500 MHz (Extension 2).



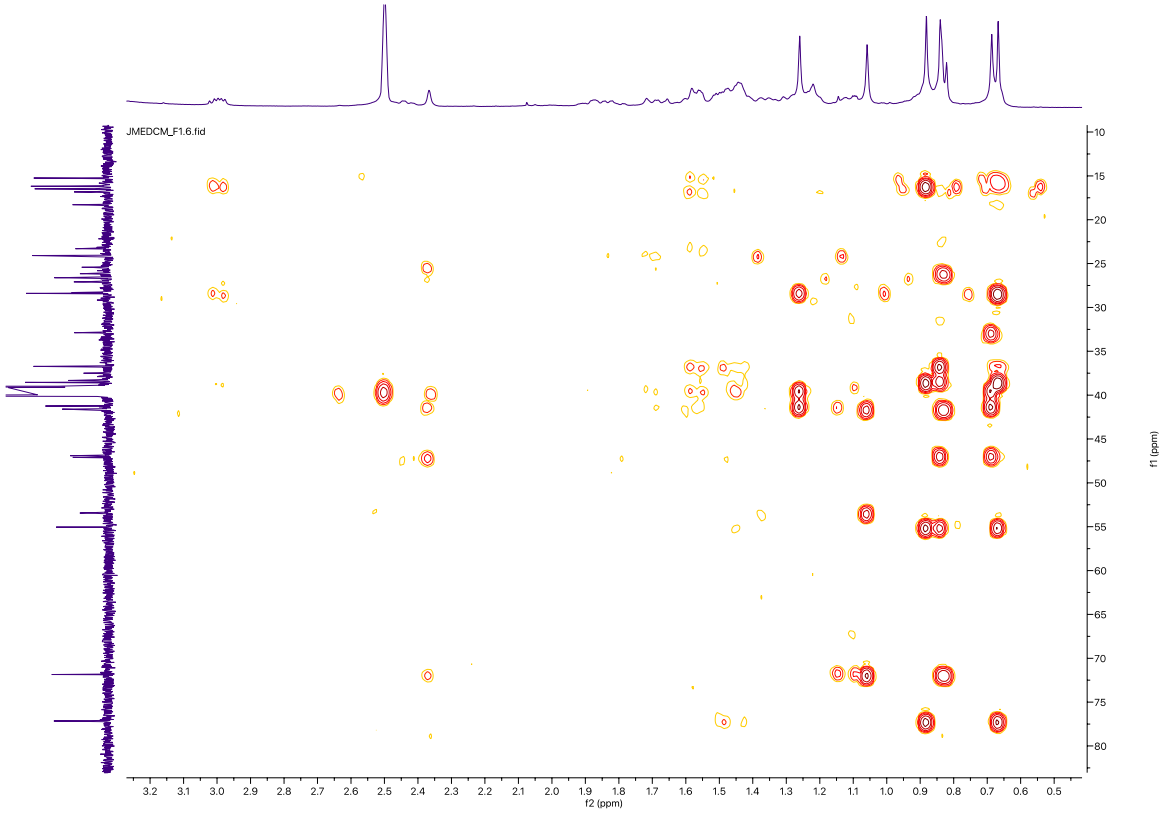
-Figure S11. HSQC spectrum of JMEDCM_F1 in DMSO 500 MHz.



-Figure S12. HSQC spectrum of JMEDCM_F1 in DMSO 500 MHz (Extension 1).



-Figure S13. HMBC spectrum of JMEDCM_F1 in DMSO 500 MHz.



-Figure S14. HMBC spectrum of JMEDCM_F1 in DMSO 500 MHz (Extension 1).

Elemental Composition Report
Multiple Mass Analysis: 122 mass(es) processed – displaying only valid results
Tolerance = 10.0 PPM / DBE: min = - 1.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Odd and Even Electron Ions
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Element Used:
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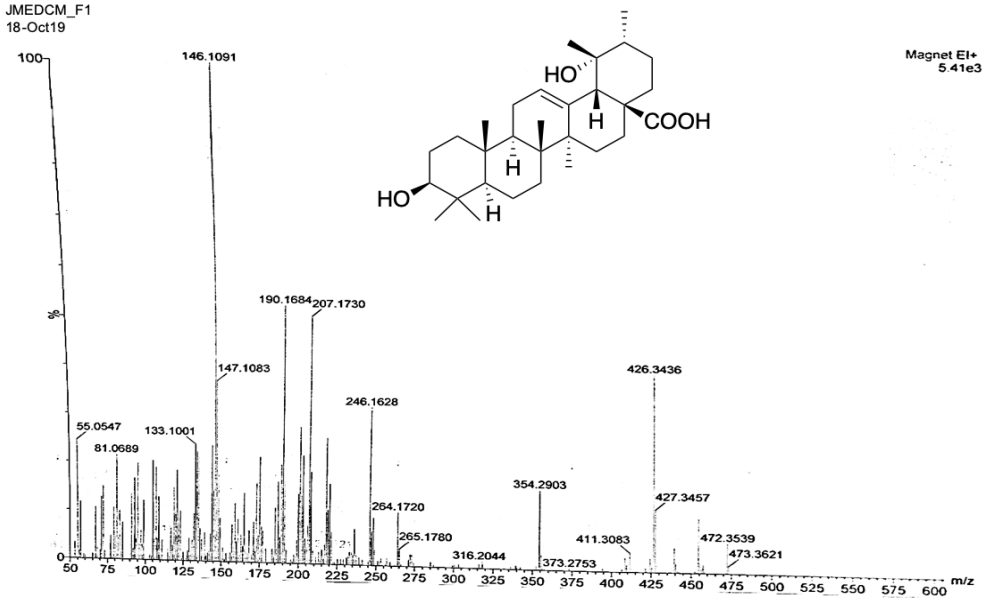
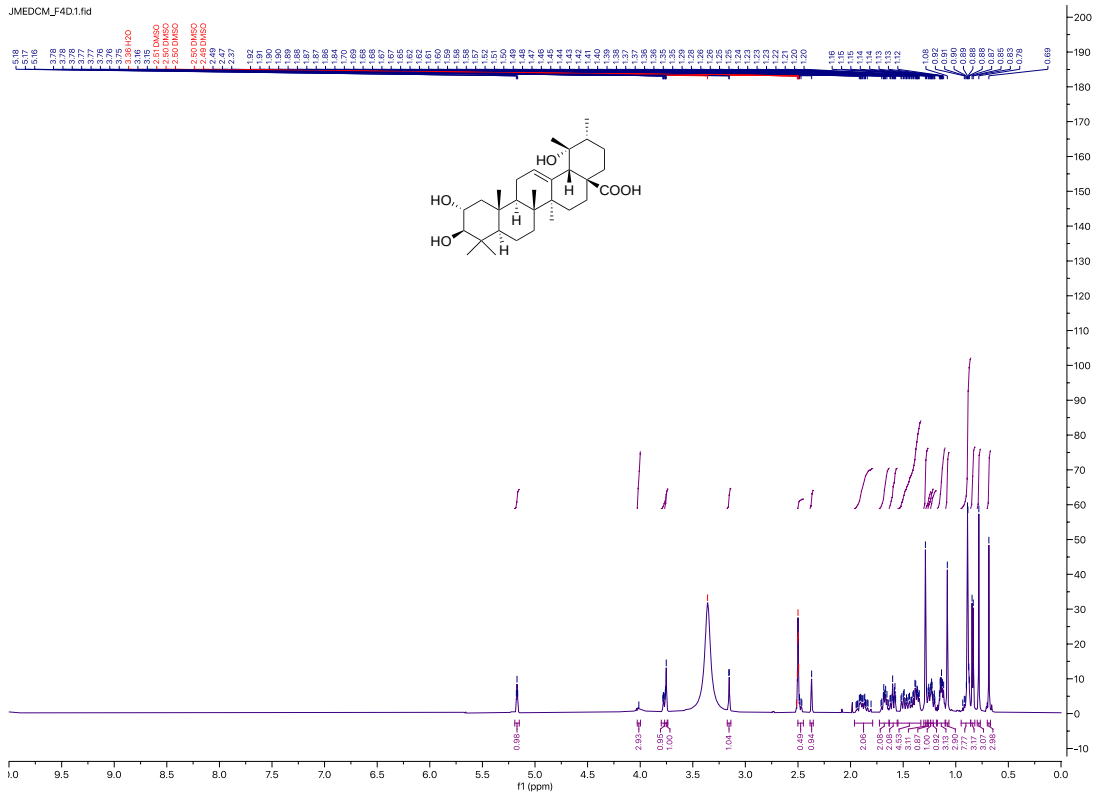
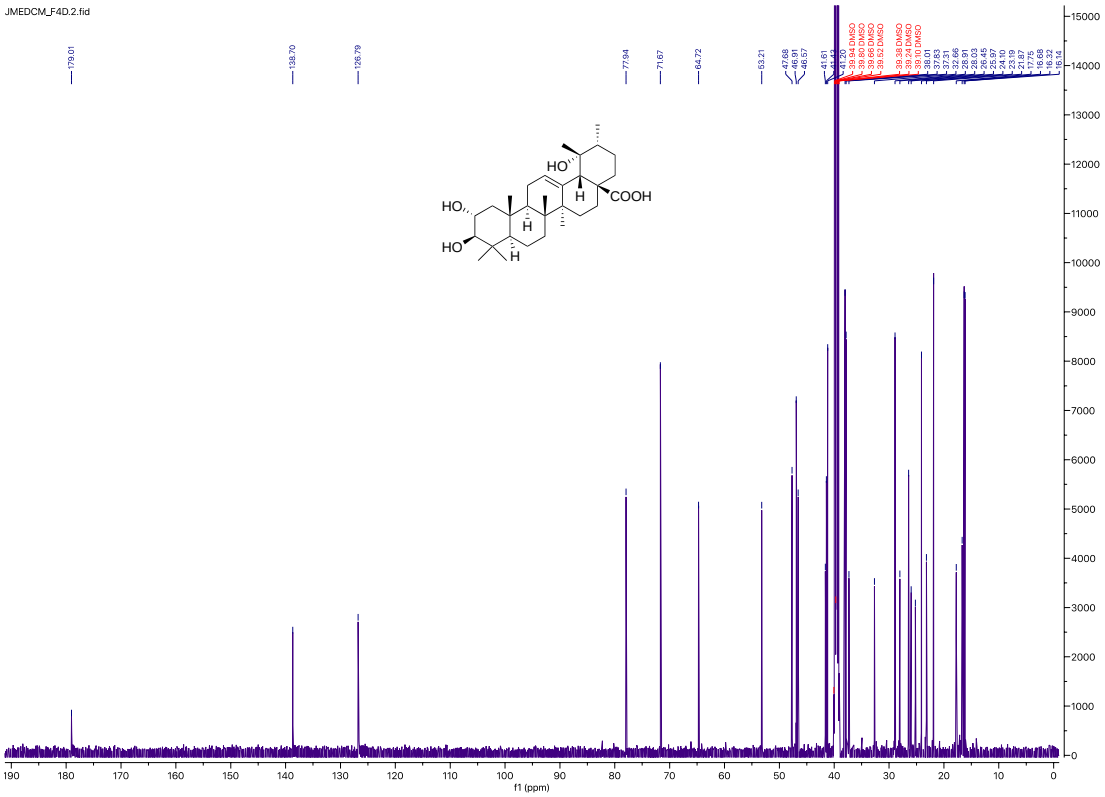
JMEDCM_F1
18-Oct19



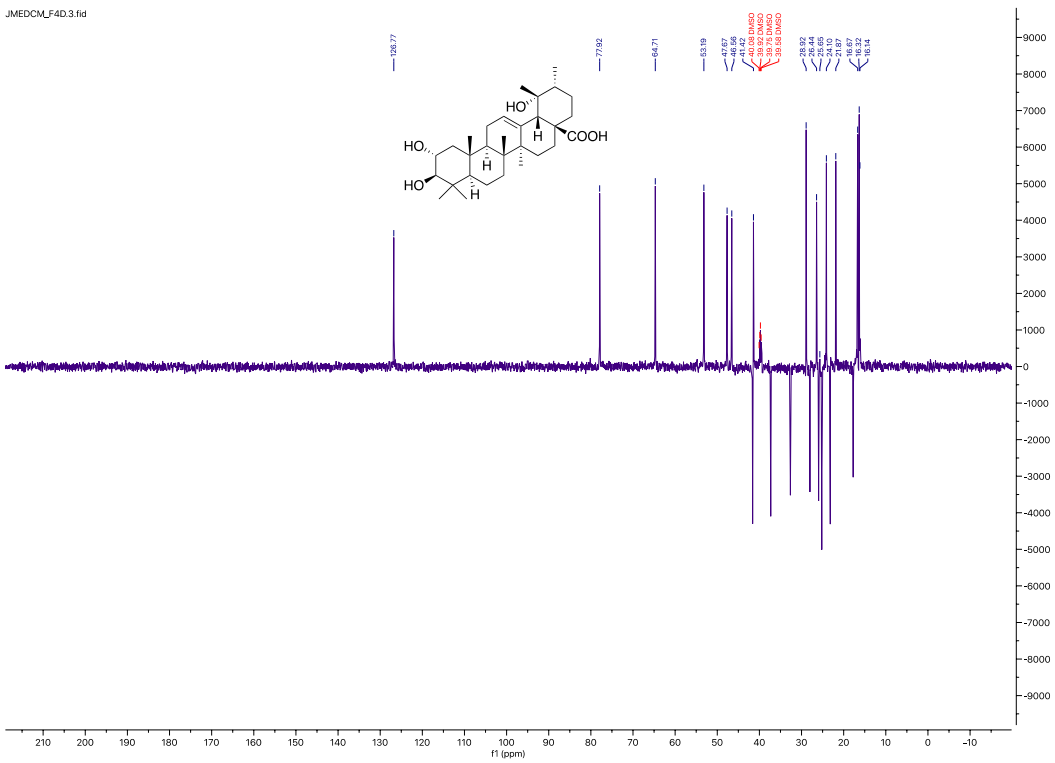
-Figure S15. HRMS spectrum of JMEDCM_F1.



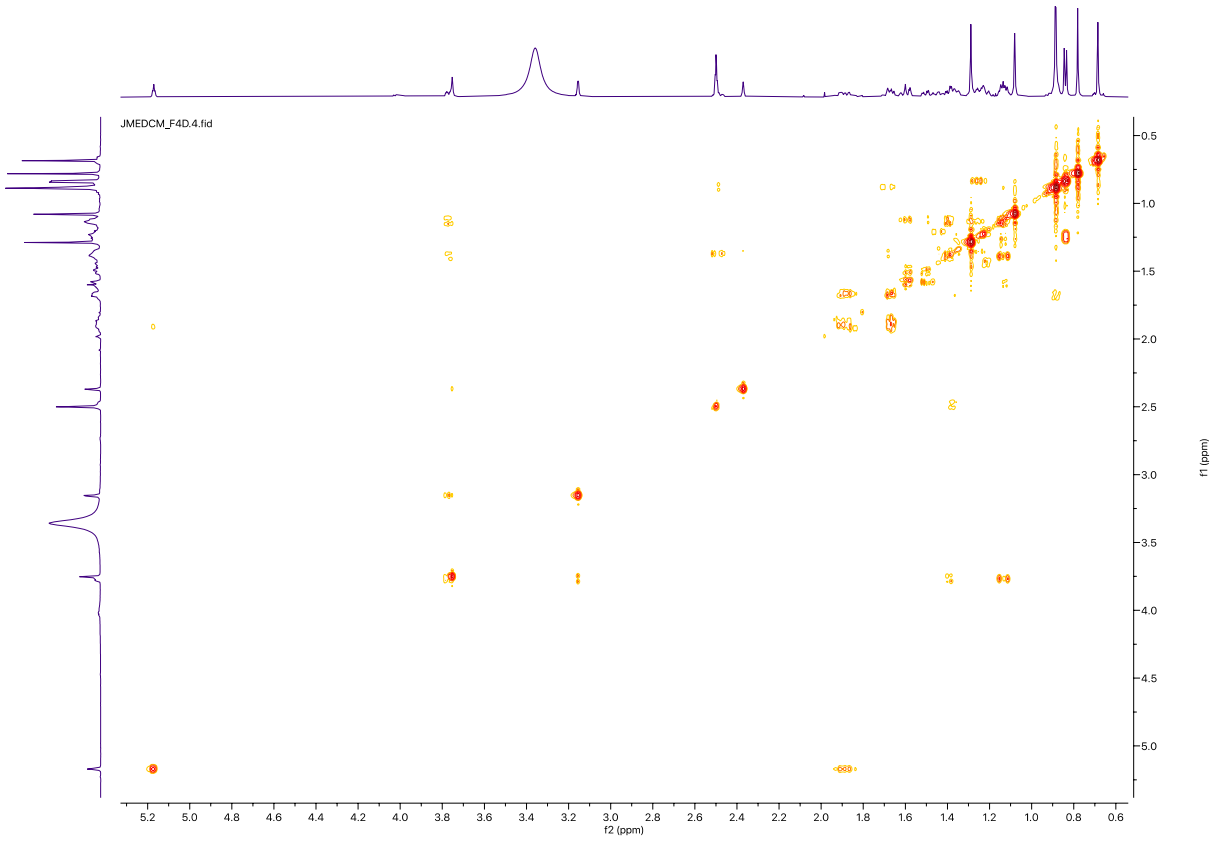
-Figure S16. ^{1}H-NMR spectrum of JMEDCM_F4D in DMSO 600 MHz.



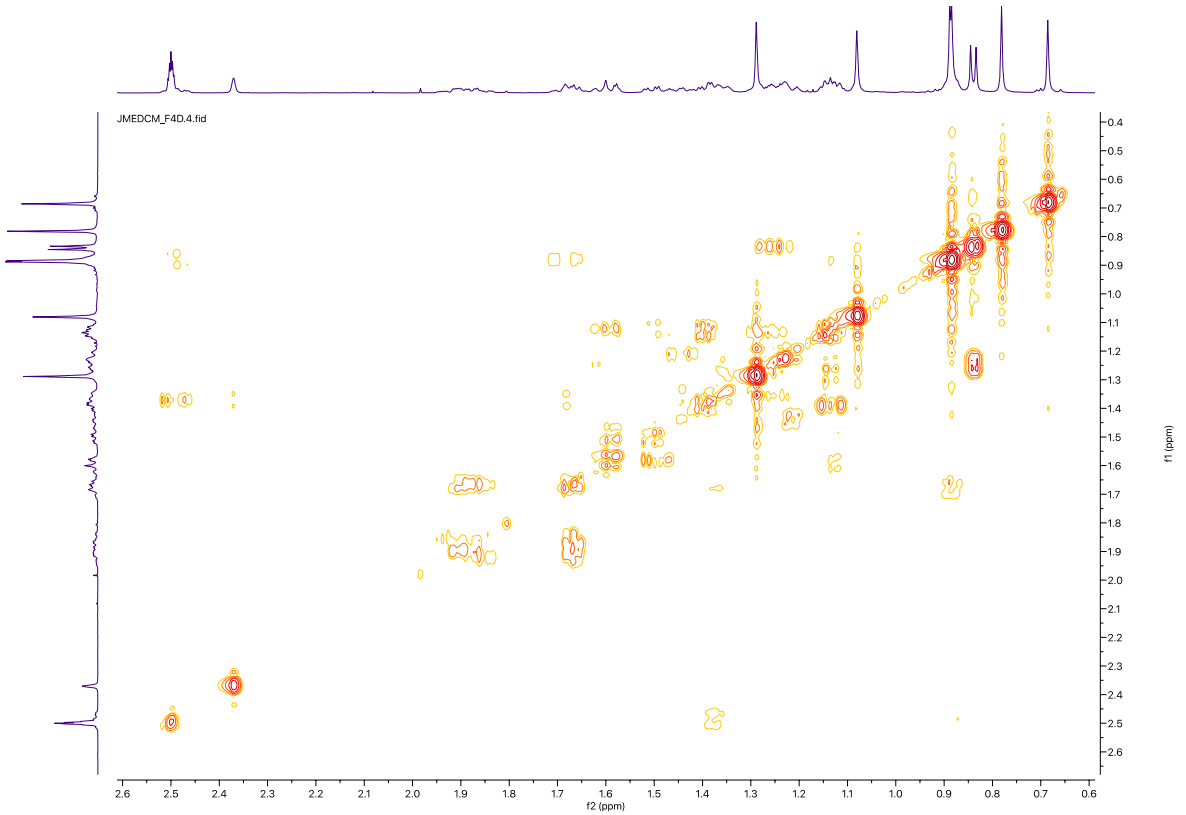
-Figure S17. ^{13}C-NMR spectrum of JMEDCM_F4D in DMSO 151 MHz.



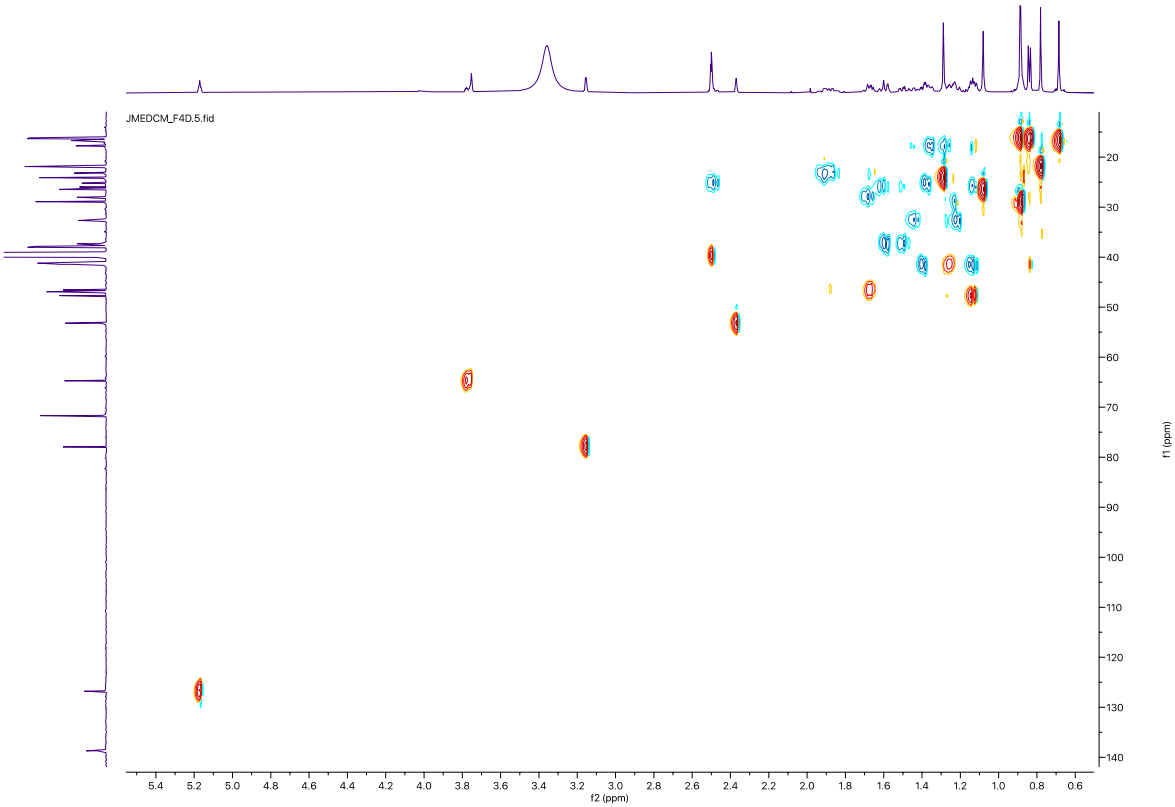
-Figure S18. DEPT-135 spectrum of JMEDCM_F4D in DMSO 151 MHz.



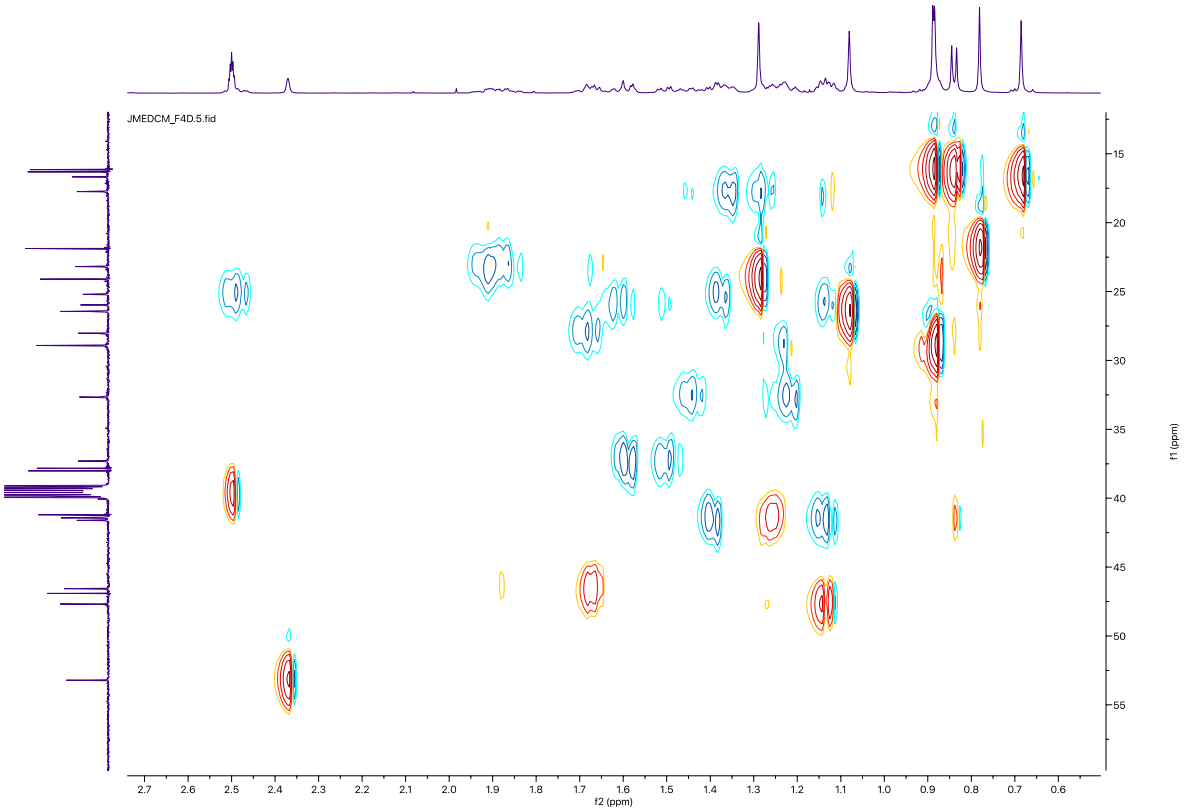
-Figure S19. ^{1}H-^{1}H COSY spectrum of JMEDCM_F4D in DMSO 600 MHz.



-Figure S20. ^{1}H-^{1}H COSY spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 1).

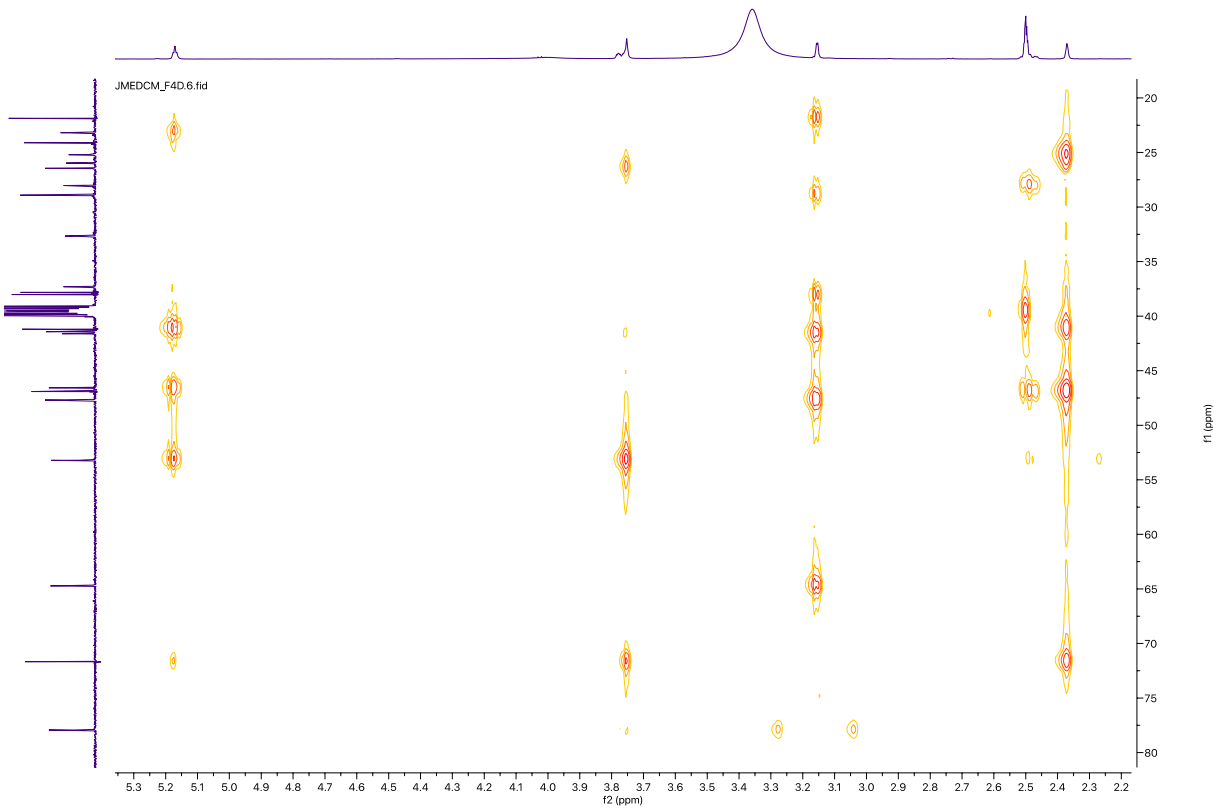


-Figure S21. HSQC spectrum of JMEDCM_F4D in DMSO 600 MHz.

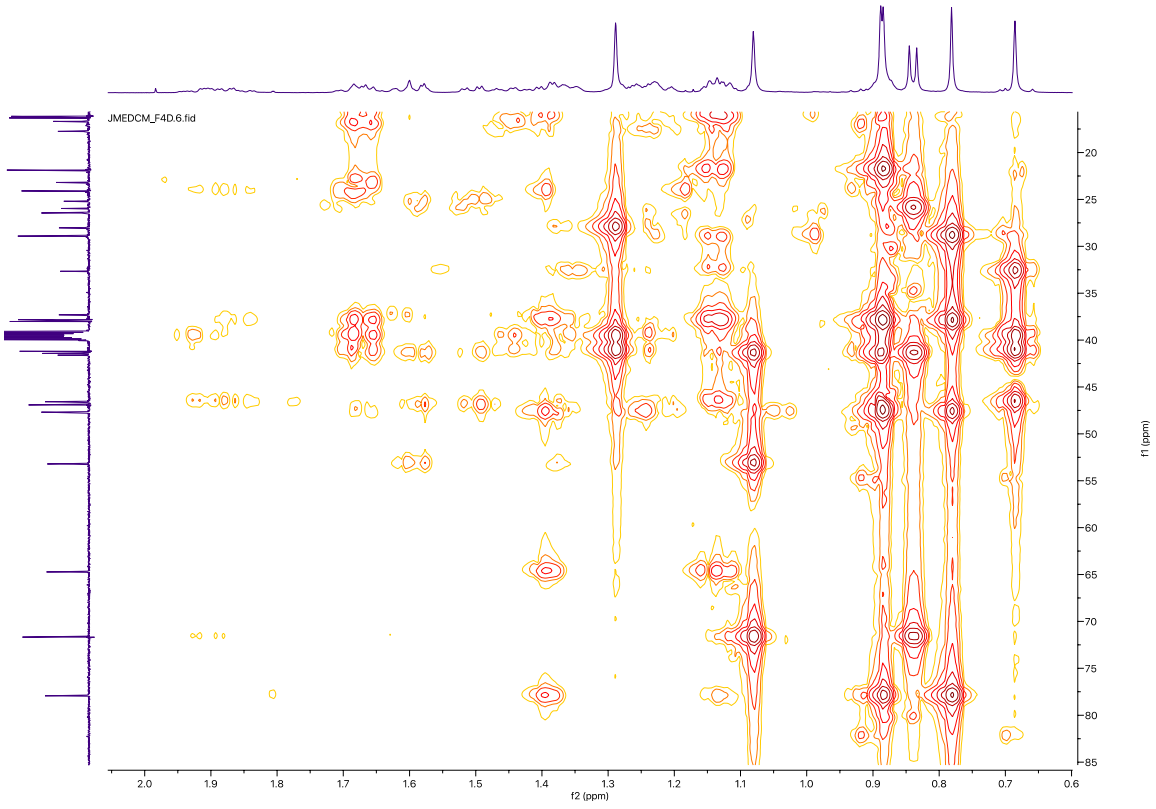


-Figure S22. HSQC spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 1).

-Figure S23. HMBC spectrum of JMEDCM_F4D in DMSO 600 MHz.



-Figure S24. HMBC spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 1).



-Figure S25. HMBC spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 2).

Elemental Composition Report
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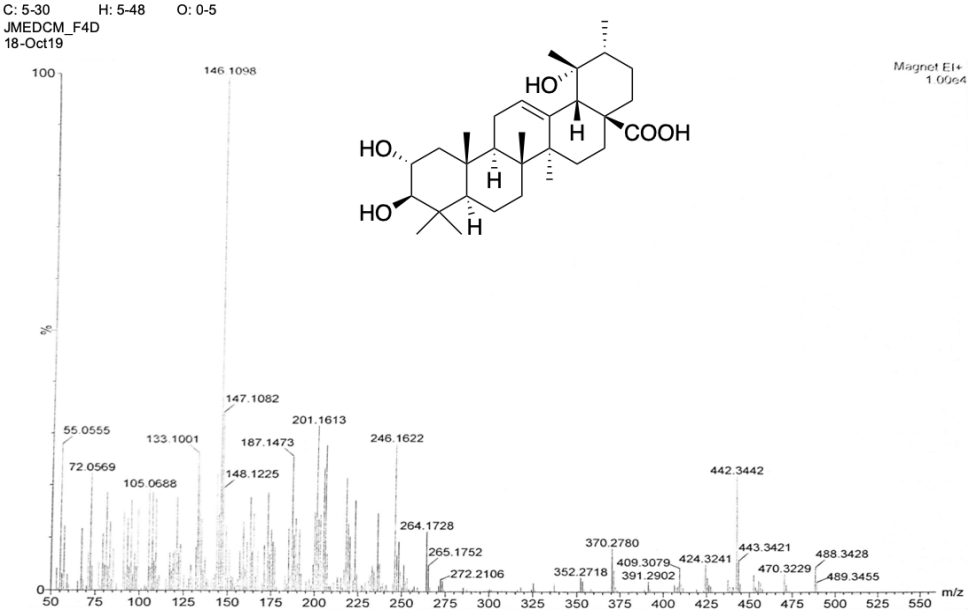
Tolerance = 10.0 PPM / DBE: min = - 1.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Odd and Even Electron Ions
4057 formula(e) evaluated with 80 results within limits (all results (up to 1000) for each mass)
Element Used:
C: 5-30 H: 5-48 O: 0-5
JMEDCM_F4D
18-Oct19



-Figure S26. HRMS spectrum of JMEDCM_F4D.

- Table S1. XTT viability assay of *J. macrantha* extracts and compounds against a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic (1% O_2) conditions. Data presents the means (±SD) of three separate experiments performed in triplicate. Docetaxel was used as positive control.

Samples	CC_{50}			
	PBMCs	SK-MEL-28	A549	U-373 MG
n-Hexane extract[(a)]	97.09±1.82	29.36±1.27	51.97±1.97	48.03±1.77
CH_2Cl_2 extract[(a)]	>100±1.00	11.89±1.04	36.88±1.05	29.95±1.09
MeOH extract[(a)]	>100±1.61	9.05±1.13	21.76±1.03	12.05±1.02
Aqueous extract[(a)]	>100±1.45	88.94±1.72	>100±1.17	91.54±1.11
Compound 1[(b)]	61.23±1.07	5.01±0.02	20.80±1.13	12.70±1.10
Compound 2[(b)]	73.00±1.00	8.79±0.03	26.73±1.14	17.31±1.15
Docetaxel[(c)]	52.94±1.01	3.95±1.02	5.67±1.21	4.48±1.25

(a)=CC_{50} in μg/mL; (b)=CC_{50} in μM; (c)=CC_{50} in *n*M

- Table S2. Inhibitory effect of *J. macrantha* extracts and compounds on NF-κB activation in a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic (1% O_2) conditions. The results are the means (±SD) of three separate experiments performed in triplicate. JSH-23 was used as positive control.

Samples	IC_{50}			
	PBMCs	SK-MEL-28	A549	U-373 MG
Control	11.99±0.01	12.14±0.07	12.13±0.01	12.01±0.05
DMSO	10.70±0.03	10.67±0.01	10.76±0.02	10.71±0.03
n-Hexane extract[(a)]	5.19±0.01	5.03±0.01	5.99±0.01	5.67±0.02
CH_2Cl_2 extract[(a)]	4.46±0.02	2.78±0.04	4.39±0.02	4.55±0.01
MeOH extract[(a)]	8.85±0.01	8.65±0.03	8.87±0.01	8.80±0.02
Aqueous extract[(a)]	11.67±0.03	10.09±0.03	12.07±0.02	11.77±0.03
Compound 1[(b)]	2.70±0.01	1.05±0.02	3.63±0.01	2.55±0.02
Compound 2[(b)]	3.17±0.01	2.71±0.01	3.73±0.02	3.39±0.01
JSH-23[(b)]	7.12±0.03	7.11±0.01	7.14±0.03	7.13±0.01

(a)=IC_{50} in μg/mL; (b)=IC_{50} in μM

- Table S3. Inhibitory effect of *J. macrantha* extracts and compounds on HIF-1α in a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic conditions. The results are the means (±SD) of three separate experiments performed in triplicate. 2-MeOE2 was used as positive control.

Samples	IC_{50}			
	PBMCs	SK-MEL-28	A549	U-373 MG
Control	96.10±0.04	94.47±0.02	97.45±0.01	96.506±0.01
DMSO	93.10±0.03	90.83±0.02	95.12±0.03	93.62±0.02
n-Hexane extract[(a)]	9.05±0.03	7.72±0.03	17.07±0.01	11.30±0.02
CH_2Cl_2 extract[(a)]	6.19±0.01	5.83±0.01	13.14±0.02	9.17±0.01
MeOH extract[(a)]	88.01±0.01	79.00±0.01	87.02±0.02	85.01±0.01
Aqueous extract[(a)]	90.05±0.01	87.12±0.01	92.00±0.01	90.13±0.02
Compound 1[(b)]	47.88±0.02	3.01±0.02	9.97±0.01	6.34±0.02
Compound 2[(b)]	60.02±0.02	3.78±0.02	10.25±0.01	8.85±0.02
2-MeOE2[(b)]	0.45±0.01	0.51±0.02	0.49±0.02	0.47±0.03

(a)=IC_{50} in μg/mL; (b)=IC_{50} in μM

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