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REVIEW

Chemistry, Pharmacology and Health Benefits of Anthocyanins

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Anthocyanins are naturally occurring molecules belonging to the flavonoid class characterized by the presence of chromophores. Apart from their well-known antioxidant activity, they show a wide variety of health-promoting properties for human health, ranging from cytoprotective, antimicrobial and antitumour activities to neuroprotective, anti-obesity and lipidomic potential, properties for which anthocyanins have been prescribed as medicines in several countries for thousands of years. Despite this, these phytochemicals have received less attention than other flavonoids, and there is still a gap in the literature, particularly regarding pharmacological and toxicological aspects. Moreover, epidemiological evidence suggests a direct correlation between anthocyanin intake and a lower incidence of chronic and degenerative diseases. In light of this, the aim of this review is to cover the current literature on anthocyanins, their biological *in vitro* and *in vivo* effects and their potential therapeutic applications, as well as their bioavailability and pharmacokinetics, all of which are essential to gain a better understanding of their biological effectiveness and potential toxicity. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: anthocyanins; dietary sources; pharmacokinetics; health effects; toxicological aspects.

INTRODUCTION

Anthocyanins (from Greek *νθός* (*anthos*) = flower and *κυανός* (*kyanos*) = blue) constitute the largest and probably the most important group of water-soluble plant pigments. They belong to the widespread flavonoid group of polyphenols, which are responsible for the blue, purple and red colour of many plant tissues. They play an important role in attracting animals, thereby promoting seed dispersal and pollination and, by absorbing light, contribute to protecting plants from ultraviolet (UV)-induced damage (Castañeda-Ovando *et al*., 2009). Anthocyanins are polyhydroxy and polymethoxy 2-phenylbenzopyrylium derivatives constituted by a core called anthocyanidin bound to several glycosidic moieties (glucose, galactose, xylose, arabinose, fructose and rhamnose) attached at the C3, C5 or C7 positions (Smeriglio *et al*., 2014). Glycosylation confers water solubility to the parent anthocyanidins, for example, the acylation of the sugar residues with cinnamic acid or aliphatic acids that improves anthocyanin stability (Mazza *et al*., 2004). Common anthocyanidins in vascular plants are pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp). The most widespread in nature among these are the three glycosylated non-methylated anthocyanidins (Cy, Dp and Pg), representing about 70% of pigmented tissues. The content of the six most common anthocyanins in the edible parts of plants differs greatly, with Cy accounting for about 50%, followed by Pg, Pn, Dp, Pt and Mv. More than 600 anthocyanins are estimated to have been found in nature, and this number is still expected to grow (Kong *et al*., 2003; Lucioli, 2012; Castañeda-Ovando *et al*., 2009). These natural compounds are commonly found in the human diet, particularly in red, blue or purple fruits and vegetables in which they are normally observed in concentrations ranging from 0.1% up to 1.0% of dry weight (Pojer *et al*., 2013). Human daily intake of anthocyanins is quite variable and highly dependent on eating habits. Wu *et al*. (2006) estimated the average anthocyanin intake in US adults to be 12.5 mg/day, while Zamora-Ros *et al*. (2011a, 2011b) calculated the anthocyanin intake in Europeans and found the total mean intake of anthocyanidins for men ranged from 19.8 (the Netherlands) to 64.9 mg/day (Italy), whereas for women, the range was 18.4 (Spain) to 44.1 mg/day (Italy). The highest consumption found in Italy can be ascribed to the Mediterranean diet, which includes red and blue fruit and vegetables as well as red wine. Many studies have linked these compounds to antioxidant, antiinflammatory and anticarcinogenic properties. In fact, an inverse correlation has been found between the consumption of diet-derived polyphenol compounds and the incidence not only of certain types of cancer but also of cardiovascular, metabolic and other degenerative diseases (Wang and Stoner, 2008a, 2008b; Zafra-Stone *et al*., 2007; He and Giusti, 2010; Tsuda, 2012). However, these findings have yet to be confirmed by animal and human clinical trials, as results of those carried out to date are still limited and controversial. It seems, from this point of view, that another important aspect to be considered is the dose of polyphenols necessary to achieve the optimum of the biological effect. Some studies have tackled this issue analysing the anthocyanin content of

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some anthocyanins-rich dietary supplements. Among them, Vlachojannis *et al.* (2015a, 2015b) estimated the daily dose for the treatment of metabolic syndrome disorders and influenza of some elderberry and chokeberry products, highlighting that some of which were inappropriate as dietary supplements owing to the low content of active principles; therefore, they were inappropriate for clinical use.

The potential pharmacological activities of many different dietary bioactive compounds are currently being studied using *in vitro* and *in vivo* models (Valenti *et al.*, 2013). From this point of view, the anthocyanins represent one of the most widely investigated of the polyphenols class. One reason is that they represent a promising alternative to the most widely used synthetic food dyes, which cause toxic effects in humans, and are thus becoming increasingly important to the food industry and to consumers (Kamiloglu *et al.*, 2015).

This review provides an overview of the latest developments and knowledge on the occurrence, dietary intake and health effects of anthocyanins focusing on pharmacological and toxicological aspects.

CHEMICAL FEATURES

Anthocyanins are glycosylated polyphenolic compounds (anthocyanidins) characterized by the presence of two benzyl rings (A and B) and a heterocyclic ring (C). The C ring is joined to the A and B rings through a three-carbon bridge. One of the most striking properties of these compounds is their colour changes in relation to matrix pH. For instance, at low pH, they appear pink, purple in neutral conditions and greenish-yellow in basics, making them natural pH indicators. If pH is very alkaline, they are colourless. These properties are due to the presence of the resonant structure of the flavylium ion (Wrolstad *et al.*, 2005) that undergoes electron transition when pH values change and is responsible for their redox properties. They are commonly glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts. The presence of so many derivatives may be due to the following: the number and position of the sugars; the number and position of hydroxyl groups; and finally, the presence of aromatic and aliphatic acids linked to the sugars (Andersen and Jordheim, 2006). Glycosylation often occurs on the hydroxyl group at C3 position, but 3,5-glycosylated and 3,7-glycosylated derivatives have also been identified (Kong *et al.*, 2003), as have 3′ and/or 5′ substitutions but less frequently. The most commonly encountered sugars are hexoses (glucose and galactose) and pentoses (arabinose, rhamnose and xylose) forming α or β linkages. Nevertheless, despite the large number of derivatives identified, only a limited number of anthocyanins derived from six anthocyanidins (Pg, Pn, Cy, Mv, Pt and Dp) are usually found in edible pigmented natural sources (leaves, fruits and flowers). Figure 1 depicts the chemical structure of these six derivatives, and a comprehensive online database is available (http://www.metabolome.jp). The presence of the flavylium ion and its peculiar electron distribution, with respect to other polyphenols, make anthocyanins highly unstable molecules both *in vitro* and *in vivo* when subjected to changes in pH, light, temperature and to oxygen and metal ion degradation. Moreover, their degradation can also be induced by intermolecular and intramolecular interactions, as well as by B-ring hydroxylation patterns (Woodward *et al.*, 2009).

BIOSYNTHESIS AND DIETARY SOURCES

The raw materials for the biosynthesis of anthocyanins are produced by the acetate and shikimate pathways. The shikimate pathway, starting from the amino acid phenylalanine, produces coumaroyl-coenzyme A (CoA) that condenses with three molecules of malonyl-CoA (obtained via the acetate pathway) to form a tetrahydroxychalcone in a reaction catalysed by chalcone synthase (Fig. 2). Chalcone isomerase converts tetrahydroxychalcone into the flavanone naringenin, which is further hydroxylated into eriodictyol by the flavonoid 3′-hydroxylase. Both compounds (naringenin and eriodictyol) are hydroxylated into the corresponding dihydroflavonols (or flavanonols), these being precursors for the production of flavan-3,4-diols (leucoanthocyanidins) following a reaction triggered by the enzyme dihydroflavonol 4-reductase. The monooxygenase flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase are the key enzymes that determine the chemical structures of anthocyanins and thus their pigmentation. The activity of both enzymes is necessary for Cy and Dp, determining the hydroxylation pattern of the B-ring (Tanaka, 2006). Anthocyanidin synthase (also called leucoanthocyanidin dioxygenase) is a 2-oxoglutarate-dependent dioxygenase that catalyses the synthesis of anthocyanidins. The conversion of anthocyanidins into anthocyanins is family-dependent or species-dependent and characterized by a high degree of diversity. They are commonly 3-*O*-glucosylated by the

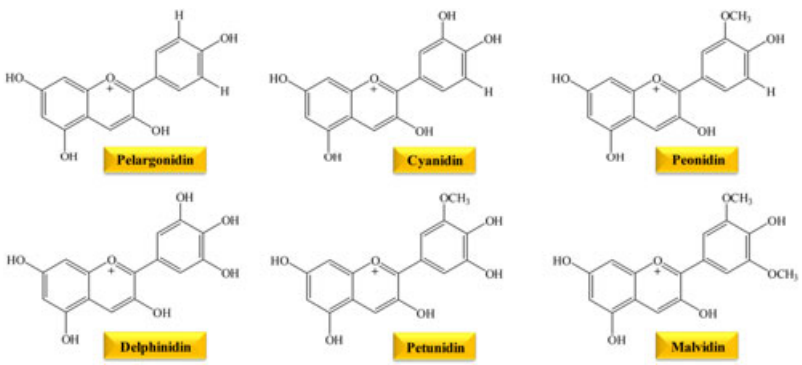

Figure 1. Chemical structure of the six most common anthocyanidins: precursors of anthocyanins.

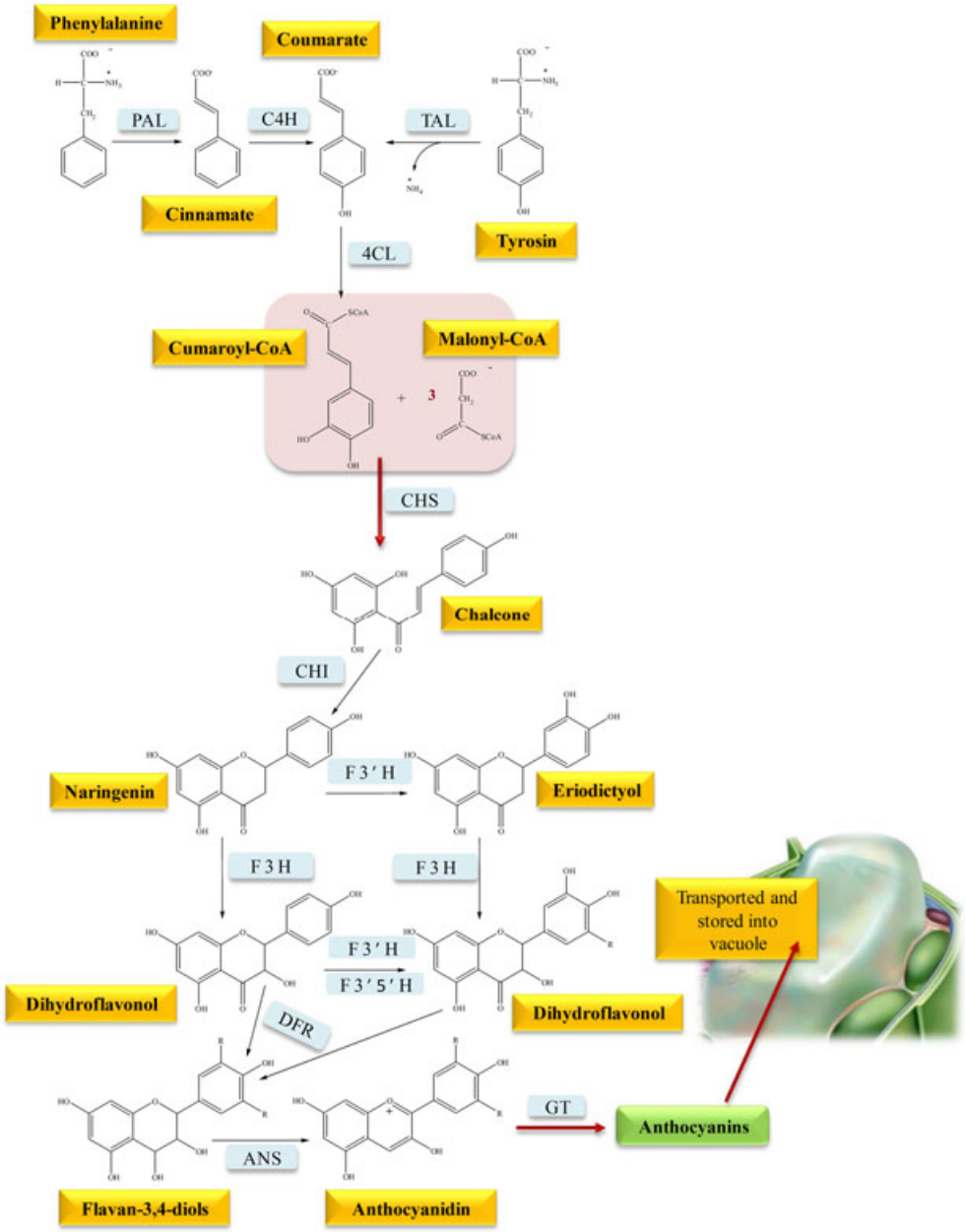

Figure 2. Schematic of the major branch pathways of anthocyanins biosynthesis, starting from general phenylpropanoid metabolism.

action of uridine diphosphate glucose (UDP-glucose) flavonoid (or anthocyanidin) 3-glucosyltransferases, which is followed by C5-glucosylation. They can be further modified by acyltransferases, mainly belonging to the BAHD family, which are able to catalyse the transfer of acylating agents (such as *p*-cinnamic, caffeic, acetic, oxalic, succinic, molonic or malic acids) to the hydroxyl group of the bound sugar. After their synthesis, anthocyanins are transported and stored in the vacuole through the following three possible mechanisms: (i) via a glutathione S-transferase-like protein and a multi-drug resistance-like protein; (ii) via vesicle-mediated mass transport; and (iii) via flavonoid/H^+-antiporter (Goodman *et al*., 2004; Zhang *et al*., 2006; Marinova *et al*., 2007a; Marinova *et al*., 2007b). Being so wide-spread, anthocyanins are one of the most common classes of secondary metabolites ingested in the daily diet, with known or proposed health-promoting benefits. The total amount of anthocyanins varies substantially across plant species and is also influenced by environmental factors such as temperature, light and altitude (Wu et al., 2006). Flowers and fruits are the richest sources, but they are also present in stems, leaves and storage organs (Delgado-Vargas & Paredes-Lopez, 2003). Coloured fruits such as peaches, berries, pomegranates, cherries, plums and grapes as well as many dark-coloured vegetables (black beans, red radishes, red onions, eggplants, red cabbage, purple corn and purple sweet potatoes) (Wu *et al*., 2006) are all rich in anthocyanins. These molecules are present not only in natural sources but also in their processed form in foods and beverages such as red wine, juices, yogurt and jelly. The most common anthocyanins in edible foods are the glycosides of the aforementioned six most widespread anthocyanidins; for instance, a glass of red wine contains glycosides of Cy, Dp, Pn, Pt and Mv (Waterhouse, 2002). Given that glucose is the most common sugar as a glycosylating agent, the 3-*O* substituted derivatives are

Table 1. Principal anthocyanins in human dietary sources

Compounds	Amount (g/100 g FW)	Sources	Reference
Malvidin-3-glucoside	10.0	Red wine	Neveu *et al.*, 2010
	39.3	Black grapes	
Cyanidin-3-glucoside	794.1	Elderberries	Jakobek *et al.*, 2007;
	405.0	Bilberries	Neveu *et al.*, 2010;
	138.7	Blackberries	Ogawa *et al.*, 2008;
	110–40	Apple	USA database;
	25.1	Blackcurrants	Pojer *et al.*, 2013;
	3.5	Blood orange	Mulabagal *et al.*, 2007
	0.7	Strawberry	
	0.4	Red onion	
Cyanidin 3-(6″-malonylglucoside)	1.6	Red onion	Pérez-Gregorio *et al.*, 2010;
	15.0	Blood orange	Barreca *et al.*, 2014;
	200–400	Red oak leaf and Red lollo lettuce	USA database
Cyanidin-3-galactoside	557.7	Chokeberries	Tomaino *et al.*, 2010;
	370.0	Bilberries	Neveu *et al.*, 2010;
	5.9	Pistachio	Ogawa *et al.*, 2008; USA database
Cyanidinin-3-sambubioside	463.0	Elderberries	Neveu *et al.*, 2010
Cyanidin -3-rutinoside	160.8	Blackcurrants	Neveu *et al.*, 2010
	8.9	Blackberries	
Cyanidin-3-arabinoside	252.8	Chokeberries	Neveu *et al.*, 2010
Cyanidin 3,5-diglucoside	24–236	Pomegranate juices	Mousavinejad *et al.*, 2009;
	30.0	Red cabbage	Gachovska *et al.*, 2010
Cyanidin 3-(sinapoyl)diglucoside-5-glucoside	31.0	Red cabbage	Gachovska *et al.*, 2010
Cyanidin 3-(sinapoyl) (sinapoyl)-diglucoside-5-glucoside	28.0	Red cabbage	Gachovska *et al.*, 2010
Cyanidin 3-(p-coumaroyl)-diglucoside-5-glucoside	25.0	Red cabbage	Gachovska *et al.*, 2010
Peonidin-3-glucoside	365.0	Blueberries	Ogawa *et al.*, 2008
Delphinidin-3-rutinoside	304.9	Blackcurrants	Neveu *et al.*, 2010
Delphinidin-3- glucoside	86.7	Blackcurrants	Neveu *et al.*, 2010
	5.0–104.0	Pomegranate juices	Mousavinejad *et al.*, 2009
Delphinidin 3,5-diglucoside	37.0–530.0	Pomegranate juices	Mousavinejad *et al.*, 2009
Pelargonidin-3-glucoside	47.2	Chokeberries	Jakobek *et al.*, 2007;
	15.9	Strawberry	Neveu *et al.*, 2010; USA database
Pelargonidin-3-rutinoside	3.6	Strawberry	Jakobek *et al.*, 2007
Pelargonidin 3,5-diglucoside	0.7–9.0	Pomegranate juices	Mousavinejad *et al.*, 2009

the most widespread anthocyanins in nature (Andersen and Jordheim, 2006; Kong *et al.*, 2003). Table 1 summarizes the most abundant anthocyanins and their sources. In recent reports, an average anthocyanin intake of ~12.5–65.0 mg/day has been reported for US and European populations (Wu *et al.*, 2006; Zamora-Ros *et al.*, 2011a, 2011b). Nowadays, the dietary intake of anthocyanins may be considerably higher owing to the commercial availability of concentrated red fruit extracts and the substitution of synthetic colourants with these natural pigments in many soft drinks (Jing and Giusti, 2005; Giusti and Wrolstad, 2003). Moreover, regular consumption of red wine (in moderate amounts), blueberries and/or grapes markedly increases anthocyanin intake (Clifford, 2000).

PHARMACOKINETICS

The biological activities ascribed to anthocyanins are closely related to their pharmacokinetic characteristics. Recent studies indicate that anthocyanins are rapidly absorbed in the stomach and small intestine of rats (He *et al.*, 2009; Passamonti *et al.*, 2003; Talavera *et al.*, 2003) as demonstrated by the intense red colour of the stomach and small intestine tissues examined, and they appear in blood circulation and urine as intact, methylated, glucuronide and/or sulfate conjugated forms (Felgines *et al.*, 2005; Mazza *et al.*, 2002a, 2002b; Wu *et al.*, 2002; Felgines *et al.*, 2003; Kay *et al.*, 2005; Matsumoto *et al.*, 2006a, 2006b; Mullen *et al.*, 2010; Cooney *et al.*, 2004; Talavéra *et al.*, 2005). Animal model studies indicate that anthocyanins appear in the bloodstream within a few minutes (6 to 20 min) after consumption and reach maximum blood levels after 15 to 180 min, depending on the individual compound and the food matrix, with plasma concentration observed in the nM to low μM range (Matsumoto *et al.*, 2001, 2005; Matsumoto *et al.*, 2006a, 2006b; Passamonti *et al.*, 2003; Wu *et al.*, 2004; Ichiyanagi *et al.*, 2005; Ichiyanagi *et al.*, 2006; McGhie and Walton, 2007). Although absorption through the gastric mucosa is unusual for

nutrients, these characteristics are consistent with previous observations showing anthocyanins to be absorbed at this level (Passamonti *et al*., 2003; Talavera *et al*., 2003). This observation has been confirmed by *in situ* gastric administrations of anthocyanins demonstrating, for example, that malvidin-3-glucoside appears in the plasma within 6 min, as well as by some anthocyanin glucosides and galactosides that were measurable in portal and systemic plasma (Passamonti *et al*., 2003; Talavera *et al*., 2003). These and other studies, in to supporting previous findings, suggest a plausible absorption mechanism for anthocyanins, that is, mediated by an organic anion membrane carrier bilitranslocase located in the stomach (Passamonti *et al*., 2002; Nicolin *et al*., 2005). Some animal model studies have also investigated the absorption of anthocyanins through the small intestine following *in situ* perfusion administration (Talavéra *et al*., 2004). The authors found anthocyanin absorption to be higher in jejunum tissue with respect to duodenal tissue in which it was limited and to the ileum or colon in which it was totally absent (Matuschek *et al*., 2006).

Anthocyanins, being highly water-soluble molecules, are characterized by low absorption through passive diffusion (Hollman, 2004) in contrast with anthocyanidins that are more hydrophobic and passively diffuse across the mucosal epithelium (Hollman, 2004; Arts *et al*., 2004). Therefore, the absorption of anthocyanins requires either a specific active transport mechanism to transport glycosides across the intestine wall or needs to be hydrolysed to its aglycone form in the small intestine through the action of specific enzymes (Manach *et al*., 2005; Kay, 2006). Moreover, it seems, like other flavonoids (Walton *et al*., 2006; Passamonti *et al*., 2009; Kurilich *et al*., 2005), that sodium–glucose cotransport enables intact glycosides to be transported across enterocyte membranes (Mülleder *et al*., 2002). A recent kinetic study of anthocyanin transport using a gastric cell line model MKN-28 also suggested that anthocyanins are absorbed at the gastric barrier by a saturable mechanism (Fernandes *et al*., 2012). This apparent saturation supports the involvement of carriers in anthocyanin absorption.

In rats, anthocyanins appear to have a high organotropism ($T = 0.36$ min) (Vanzo *et al*., 2011), although their availability to target tissues appears limited (He and Giusti, 2010). For example, in rats fed on a blackberry anthocyanin-rich diet for 15 days, the distribution of total bioavailable anthocyanins detected was 10.13% in stomach tissue, 89.30% in the jejunum, 0.06% in the liver, 0.48% in the kidney and 0.04% in the brain (Talavéra *et al*., 2005). The concentrations observed were at a level consistent with pharmacological activity. A subsequent *in vivo* study by Kalt *et al*. (2008) showed that anthocyanins can readily cross the blood–brain barrier encountered at the cortex and cerebellum, as well as that of the liver. They also observed anthocyanin accumulation in the eyes, suggesting that these compounds also readily cross the blood–retinal barrier, which could justify the potential health effects of these substances to these areas.

Pharmacokinetic evidence suggests that plasma concentrations of anthocyanin glycosides and glucuronide derivatives are prominent between 0 and 5 h, while between 6 and 24 h, an increase in methylation occurs, suggesting that anthocyanin bioactivity is probably altered over time as a result of metabolic transformation. The metabolites persist in the urine for up to 24 h and may retain their basic anthocyanin structure (Kay *et al*., 2004; Kay *et al*., 2005).

It is known that anthocyanins are rapidly hydrolysed by intestinal microflora with cleavage of the protective 3-glycosidic moiety; the presence of a glucose moiety rather than a galactose or arabinose moiety seems to make them more bioavailable (Milbury *et al*., 2010). Although the derived compounds are highly unstable molecules under any conditions, under physiological conditions (neutral pH), they are spontaneously degraded into several monomeric phenolic acids and aldehydes, for example, protocatechuic acid, as shown by Tsuda *et al*. (2003) and phloroglucinol aldehyde (Williamson *et al*., 2009; Keppler and Humpf, 2005; Sesso *et al*., 2007; Klatsky, 2001). These metabolites may contribute to the bioavailability of anthocyanins and may be responsible for their antioxidant and health effects (Aura *et al*., 2005; Manach *et al*., 2005; Vitaglione and Donnarumma, 2007; Crozier *et al*., 2009). Once in circulation, the metabolites can be subjected to phase II metabolism with further conversions in the liver by drug detoxification enzymes (COMT, SULT1 and UDPGT).

A study carried out by González-Barrio and coworkers (2010) showed that after consumption of 300 g of raspberries by healthy human volunteers and subjects with an ileostomy, no detectable quantities of native anthocyanins or their metabolites were found in plasma from the healthy volunteers. The three main raspberry anthocyanins were found in the urine of both groups 0–7 h after ingestion in quantities $<0.1\%$ of ingested doses, suggesting poor intestinal absorption.

This evidence was confirmed also by Pérez-Jiménez *et al*. (2010) that in focusing on urinary metabolites as biomarkers of polyphenol intake in humans, they highlighted a weaker recovery for anthocyanins (0.06–0.2%) and weaker correlation with dose, suggesting that they are not suitable as biomarkers of intake.

In contrast, another study demonstrated that uptake of black raspberry anthocyanins reached 7.5% of the ingested dose in the small intestinal tissue, a much higher concentration with respect to the reported bioavailability based on plasma and urine values (He *et al*., 2009). Several *in vivo* studies suggest that the food matrix can affect the pharmacokinetics of anthocyanins. In particular, it has been shown that phytic acid contained in the hulls of nuts, seeds and grain increases the bioavailability of blackcurrant anthocyanins (Wallace, 2011). This compound is responsible for reducing gastrointestinal mobility and slows the passage of anthocyanins through the stomach, duodenum and jejunum, lengthening contact time of anthocyanins at this level and thus favouring their absorption. Results from rat and human plasma found urinary anthocyanin levels to be enhanced by phytic acid, delaying peak excretion 4–8 h post-ingestion and increasing anthocyanin recovery fourfold to fivefold (Lucioli, 2012).

Another important aspect to consider in the evaluation of anthocyanin bioavailability concerns individual variations in the xenobiotic metabolism. Indeed, several human polymorphisms of the main enzymes involved in the biotransformation mechanisms

of anthocyanins, such as variations of human gut microbiota, have been identified (Lampe and Chang, 2007).

Recently, the human colonic adenocarcinoma (Caco-2) cell line has proven to be a good alternative to animal studies representing the most common *in vitro* model of the small intestine for predicting intestinal absorption of anthocyanins. However, the bioavailability of anthocyanins may be underestimated because the metabolites formed in the course of digestion could be responsible for the health effects associated with these compounds (Kamiloglu *et al*., 2015). The Caco-2 cell model is also useful to study the molecular mechanisms that underlie anthocyanin absorption. Nevertheless, many studies analysing pure standards or anthocyanin-rich extracts derived from plants and foods at concentrations that showed a response but did not find any match with the actual concentrations or anthocyanin typologies to which real plasma and target tissues are exposed *in vivo*. In view of this, the use of combined *in vitro* digestion and Caco-2 cells could be a better approach (Kamiloglu *et al*., 2015).

Based on the aforementioned, the results would appear inconsistent, and although several *in vitro* and *in vivo* studies have been carried out, further more accurate studies using better experimental designs are needed to better understand the pharmacokinetics of anthocyanins.

BIOLOGICAL ACTIVITIES AND HEALTH EFFECTS

Epidemiological studies have highlighted an inverse correlation between a high consumption of polyphenols and the incidence of some chronic diseases. Among these, it seems that anthocyanins play an important role because they are the most abundant polyphenols in fruits and vegetables and possess potent antioxidant activity (He and Giusti, 2010). Numerous *in vitro* and *in vivo* studies suggest that anthocyanins may be positively implicated in human health. They exert different biological effects, and it seems that the consumption of foods such as juices or other formulations rich in these compounds may be correlated with antidiabetic and anti-obesity effects. These compounds may also be useful as neuroprotective agents (Prior and Wu, 2006; Tsuda, 2012) in reducing inflammation and exerting cardiovascular protection (Mazza, 2007; He and Giusti, 2010) as well as in preventing and inhibiting cancer growth (Cooke *et al*., 2005; Wang and Stoner, 2008a, 2008b; Thomasset *et al*., 2009). Some of the beneficial effects ascribed to food-derived anthocyanins are summarized in Table 2.

To date, 26 clinical trials (16 completed and 10 ongoing) about the health effects of anthocyanin are available, eleven of which about human health. These are carried out mainly in Europe and America (16 and 13, respectively) and evaluate the anthocyanins' role in the evolution of different diseases such as metabolic disorders, endocrine diseases and inflammation (www.clinicaltrial.gov). Despite low adverse effects and high health potential of anthocyanins for various diseases, only one clinical trial is available; therefore, in order to better clarify the role of these substances in this broad spectrum of health effects, future clinical trials were strongly recommended.

Antioxidant activity

Reactive oxygen species (ROS) are physiologically produced and are important to the immune system, cell signalling and many other body functions. However, if ROS are produced in excess, the body's oxidative balance is altered, and this promotes cellular damage leading to degenerative diseases such as inflammation, ageing, cardiovascular disease, cancer and metabolic disorders (Allen and Tresini, 2000). Anthocyanins possess an antioxidant power superior to other conventional antioxidants like α-tocopherol (Wang *et al*., 1997; Fukumoto and Mazza, 2000) trolox and catechin. (Kahkonen and Heinonen, 2003). This derives from the chemical structure of the molecule and, particularly, the number of hydroxyl groups, the catechol moiety in the B ring, the oxonium ion in the C ring, hydroxylation and methylation patterns and acylation and glycosylation (Yang *et al*., 2011). Glycosylation, in particular, seems to decrease antioxidant and free radical scavenger activity compared with the aglycone form, reducing the ability of anthocyanin radicals to delocalize electrons (Wang and Stoner, 2008a, 2008b). Dp and Cy anthocyanidins have been found to be the most active compounds followed by Mv, Pn, Pg and Pt (Lucioli, 2012). However, we must not forget that fruits and vegetables are rich sources of other phytochemicals and vitamins that could interact synergistically or antagonistically with anthocyanins, thereby enhancing or decreasing the antioxidant activity of these compounds (Pojer *et al*., 2013; Bolling *et al*., 2011). The antioxidant activity of 14 anthocyanins and their glycosylated derivatives has been evaluated using a widely accepted antioxidant assessment method, the oxygen radical absorbance capacity (ORAC) assay (Wang *et al*., 1997). Cy-3-glucoside (Cy-3-Glu) showed the highest ORAC value of these and Pg the lowest. Because Cy and its glycosides represent one of the major groups of naturally occurring anthocyanins, their antioxidant properties have been investigated in depth. Cy-3-Glu and its aglycone have been shown to have similar antioxidant potency to vitamin E (α-tocopherol) in different *in vitro* experimental models (Tsuda *et al.* 1994); they exert a protective effect on DNA cleavage, a dose-dependent free radical scavenging activity and significant inhibition of xanthine oxidase activity (Acquaviva *et al*., 2003). Cy-3-Glu has been shown to have protective effects *in vitro* on human umbilical vein endothelial cells affected by endothelial dysfunction and vascular failure induced by the peroxynitrite radical (Serraino *et al*., 2003). Two *in vitro* studies on human keratinocytes (HaCaT) demonstrated that Cy-3-Glu could successfully be employed as a skin photoprotective agent against UVA and UVB radiation (Tarozzi *et al*., 2005; Cimino *et al*., 2006). This observation was confirmed by Giampieri *et al.* (2012) with their observation that anthocyanins are able to protect human dermal fibroblasts against UVA radiation, increasing cellular viability and diminishing DNA damage. It has been demonstrated that Cy-3-Glu is also able to reduce ROS production and to inhibit protein and DNA synthesis caused by aflatoxin B1 and ochratoxin A in human hepatoma (Hep G2) and in CaCo-2 cell lines as well as in human fibroblasts (Guerra *et al*., 2005; Russo *et al*., 2005). It is also believed that Cy-3-Glu may play a potential role in the prevention of fibrosis in chronic liver diseases

Table 2. Biological activity and health effects of some food-derived anthocyanins in *in vitro* and *in vivo* studies

Anthocyanins source	Experimental model	Dose	Treatment time	Results	References
Bilberry			*In vitro studies*		
	Isolated rat hearts under ischemia-reperfusion conditions	0.01–1 mg/L		↓ Rate of lactate dehydrogenase (LDH) ↑ Post-ischemic coronary flow ↓ The incidence and duration of reperfusion arrhythmias	Ziberna *et al.*, 2012
	HT-29 colon cancer cells	25–75 μg/mL		↓ The growth cells	Zhao *et al.*, 2004
	Gram-positive and Gram-negative	50 μL		↓ Growth	Burdulis *et al.*, 2009
			Animal studies		
	Ischemia-reperfusion hamster model			↓ Microvascular impairments ↑ Preservation endothelium ↑ Capillary perfusion	Bertuglia *et al.*, 1995
	Type 2 diabetes mouse model	27 g of BBE/kg diet		↓ Hyperglycemia ↓ Elevation of blood glucose levels ↑ Insulin sensitivity	Takikawa *et al.*, 2010
	ApcMin mice, intestinal cancer model	9 mg	12 weeks	↓ Adenoma 30%	Cooke *et al.*, 2006
	Fischer 344 male rats	3.85 g/kg diet		↓ Aberrant crypt foci by 26% to 29%	Lala *et al.*, 2006
			In vitro studies		
	Eight young healthy males	160 mg (3 times a day)	21 days	No significant effect observed	Muth *et al.*, 2000
	Double-blind placebo-controlled study with 6 Subjects		3 h	Adapted to the light within 6.5 min	Camire 2000
	50 Patients with senile cataracts	180 mg (twice a day)	4 months	Progression of cataracts was stopped in 96% of the subjects treated	Bravetti 1988
Blueberry			*In vitro studies*		
	Gram-positive and Gram-negative	50 μL		↓ Growth	Burdulis *et al.*, 2009
			In vitro studies		
	Old F344 rats	2% diet	10 weeks	↑ HSP70-mediated protection ↓ Deleterious effects of ageing	Galli *et al.*, 2006
	F344 rats	2% diet	8 weeks	↓ Inflammation ↓ Expression of IL-1b, TNF-α and NF-kβ	Shukitt-Hale *et al.*, 2008
Blueberry			*In vitro studies*		
	Ageing OXYS rats	230 mg/kg		↓ Cataract ↓ Lipid peroxidation products in blood serum	Kolosova *et al.*, 2006

(Continues)

Table 2. (Continued)

Anthocyanins source	Experimental model	Dose	Treatment time	Results	References
	Mice	27.8 mg/g	8 weeks	↓ Body weight gain	Prior *et al.*, 2008
	Mice	0.49 mg	72 days	↓ Weight gain ↓ Body fat accumulation	Prior *et al.*, 2010
		Human studies			
	16 young normal volunteers (single oral dose)	12, 24 and 36 mg	24 h	No significant effect observed	Levy and Glovinsky, 1998
	18 young normal volunteers (twice a day)	12 and 24 mg	4 days	No significant effect observed	Zadok *et al.*, 1999
Blackcurrant			*In vitro studies*		
	Staphylococcus aureus, *Escherichia coli*, *Enterococcus faecium*	10.00 mL		↓ Growth	Werlein *et al.*, 2005
		Animal studies			
	Chick with negative lenses	50 and 100 mg/kg	3 days	↓ Transient myopia ↓ Eye fatigue ↑ Dark adaptation ↑ Retinal blood flow	Lida *et al.*, 2010
	Rats with lung inflammation	10 and 30 mg/kg		↓ Inflammation	Rossi *et al.*, 2003
		Human studies			
	Double-blind, placebo controlled crossover design study with 21 slightly myopic subjects, 2 h of continuous work on a personal computer	50 mg		↓ Diopter values	Nakaishi *et al.*, 2000
	30 glaucoma patients	50 mg/day	6 months	↑ Retinal blood flow	Ohguro *et al.*, 2007
Strawberry			*In vitro studies*		
	CAL-27, KB, HT29, HCT-116, LNCaP and DU145 cancer cells	100 μg/mL		↓ Cell viability ↓ Cell growth	Zhang *et al.*, 2008
Strawberry			*Animal studies*		
	Mice	2.9 mg/g	8 weeks	↓ Body weight gain	Prior *et al.*, 2008
			Human studies		
	Eight elderly women	240 g		↑ Antioxidant capacity in serum and urine ↑ Plasma vitamin C level	Cao *et al.*, 1998
Grape			*In vitro studies*		
	HT-29 colon cancer cells	25–75 μg/mL		↓ The growth cells	Zhao *et al.*, 2004
			Animal studies		
	Rats	21 mg/kg	6 weeks	↓ Triglycerides	Al-Awwadi *et al.*, 2005

(Continues)

Table 2. (Continued)

Anthocyanins source	Experimental model	Dose	Treatment time	Results	References
				↑ HDL	
	Fischer 344 male rats	3.85 g/kg diet		Aberrant crypt foci by 26% to 29%	Lala *et al.*, 2006
	Rats (female)	10, 15 and 20 mg	20 weeks	↓ Mammary tumours	Singletary *et al.*, 2003
	Rats (female)	830 mg/L drinking water	3 weeks	↓ Final tumour mass	Jung *et al.*, 2006
	↓DMBA DNA adducts				
			Human studies		
	22 hemodialysis patients	50 mL (twice daily)	14 days	↓ Oxidized LDL ↓ Activity of NADPH oxidase ↑ HDL	Castilla *et al.*, 2008
	20 subjects	7 mL/kg	14 days	↓ Platelet aggregation ↑ NO ↓ O2-	Freedman *et al.*, 2001
	Eight men	50 g		↓ LDL oxidation	Kamiyama *et al.*, 2009
	Older adults with memory decline	6 and 9 mL/kg	12 weeks	↑ Verbal learning ↑ Cognitive function	Krikorian *et al.* 2010
	60 patients with asthenopia	85 mg (twice daily)	4 week	73% of patients ↑ symptoms	Lee *et al.*, 2005

owing to its ability to modulate hepatic stellate cell proliferation and type I collagen synthesis induced by a pro-oxidant agent (Bendia *et al.*, 2005). A range of Cy glycosides, particularly Cy-3-Glu and Cy-3-galactoside, have been found to possess higher antioxidant activity in a liposomal membrane system with respect to certain reference compounds, that is, Trolox, butylated hydoxyanisole, butylated hydroxytoluene and tert-butyl hydroxyl quinone (Adhikari *et al.*, 2005). In two antioxidant assays, Cy-3-galactoside from a cranberry extract was found to be a more powerful inhibitor of low-density lipoprotein (LDL) oxidation than other flavonoids (Yan *et al.*, 2002).

Recently, Igwe and Charlton (2016) reviewed many *in vitro* animal studies and clinical trials about the health effects of plums as an important source of polyphenols and in particular of anthocyanins. Although plums have been shown to possess antioxidant and antiallergic properties and consumption is associated with improved cognitive function, bone health parameters and cardiovascular risk factors, the level of evidence remains low particularly with respect to the study designs, so further elucidations are needed.

Cyanidin 3-galactoside was found to be the strongest superoxide radical scavenger of plum-derived polyphenols (Chun *et al.*, 2003), exerting a protective effect against toxicity induced by linoleic acid hydroperoxide in cultured human fetal lung fibroblasts (Kaneko *et al.*, 2003). Similarly, this anthocyanin was found to inhibit malonaldehyde formation (Matsufuji and Shibamoto, 2004) and to protect rat smooth muscle and hepatoma cell lines against cytotoxicity, single-strand DNA-binding protein formation and lipid peroxidation induced by tert-butyl-hydroperoxide (Lazze *et al.*, 2003).

Regarding anthocyanin activity on LDL oxidation, Abdel-Aal *et al.* (2008) observed different behaviours depending upon the type of anthocyanin involved. Cy possessed a lower inhibitory ability against LDL oxidation compared with Dp, and these differences may be due to the extra hydroxyl group at the C5-position in the Dp structure, possibly altering the release of hydrogen ions and the hydration constant (pKH) and thus increasing inhibition of copper-induced human LDL cholesterol oxidation (Abdel-Aal *et al.*, 2008).

These findings further emphasize the degree to which the antioxidant properties of anthocyanins can be influenced by their structure. However, the mechanisms by which these compounds inhibit LDL oxidation remain unclear and may be due to multiple factors, such as scavenging activity of various radical species in the aqueous phase, interaction with peroxy radicals on the LDL surface and the ability to terminate chain reactions of lipid peroxidation by scavenging lipid radicals (Kahkonen and Heinonen, 2003).

Epidemiological studies suggest that anthocyanin intake may reduce some parameters of oxidative damage (Weisel *et al.*, 2006). Individuals who consumed anthocyanin-rich food-derived products were observed to have lower oxidative DNA damage and a significant improvement in reduced glutathione content with respect to control subjects (Weisel *et al.*, 2006). A significant improvement in serum antioxidant status was found 4–24 h post-consumption of an anthocyanin-rich meal (Mazza *et al.*, 2002a, 2002b; Youdim *et al.*, 2000a, 2000b). Moreover, Mazza *et al.* (2002a, 2002b), using the ORAC test, detected a positive correlation between

postprandial serum anthocyanin concentration and antioxidant status.

In light of the aforementioned, the possible mechanisms through which anthocyanins exert their antioxidant effects include direct and indirect pathways. Anthocyanins have a direct free radical scavenging activity due to their hydrogen (electron) donation ability or may act by enhancing endogenous antioxidant defences, either by restoring or increasing the activities of antioxidant enzymes (SOD and GPx) (Toufektsian *et al*., 2008), or by acting directly on the encoding genes for the latter (Shih *et al*., 2005). Furthermore, these compounds act indirectly on the antioxidant effects by reducing the formation of DNA oxidative abducts and endogenous ROS by inhibiting NADPH oxidase and xanthine oxidase or by modifying mitochondrial respiration and the arachidonic metabolism (Pojer *et al*., 2013).

Eye health

The health benefits of anthocyanins to vision were one of the first health properties ascribed to them (Ghosh and Konishi, 2007a, 2007b). Some *in vitro* and *in vivo* studies have been carried out focused on food-derived anthocyanins. The ability of bilberry extract to modulate adverse effects induced by an auto-fluorescence pigment (A2E) on retinal pigment epithelial cells *in vitro* was evaluated, and results highlighted the ability of this extract to suppress A2E-induced photo oxidation by quenching singlet oxygen; cells also exhibited resistance to membrane permeabilization due to the detergent-like action of A2E (Kalt *et al*., 2008). The role played by anthocyanins in eyesight has also been demonstrated in a few animal studies, the results suggesting that anthocyanins can accumulate in tissues and even cross the blood–brain barrier, accumulating in particular in the eye tissue at a maximum concentration of 700 pg/g of fresh weight. In this regard, blackcurrant anthocyanins have been extensively examined in different *in vivo* models. Results showed anthocyanins to be absorbed and distributed in ocular tissues passing through the blood–aqueous and blood–retinal barriers in both animal models used (Matsumoto *et al*., 2006a, 2006b). Oral doses of blackcurrant anthocyanins in the range 12.5–50 mg were able to decrease the dark adaptation threshold in a dose-dependent manner (Nakaishi *et al*., 2000). Furthermore, bilberry anthocyanins were found to improve night vision by interacting with rhodopsin or phosphodiesterase (Pojer *et al*., 2013). However, a systematic review revealed inconsistent evidence to support the use of anthocyanins to improve night vision (Canter and Ernst, 2004), although the negative outcomes reported may have resulted from the low doses tested, the different methodologies used, the diversity of both the subjects recruited and the anthocyanin sources (Ghosh and Konishi, 2007a, 2007b). The influence of blackcurrant anthocyanins on progression of the disease open-angle glaucoma was also investigated in a randomized, placebo-controlled, double-masked trial, highlighting a statistically significant difference between the treatment groups in mean change from baseline 24 months after the start of treatment (Ohguro *et al*., 2012), inhibiting transient myopia, improving dark adaption and reducing eye fatigue (Iida *et al*., 2010). These results strongly suggest that anthocyanins are absorbed and carry out several physiological activities and confer ocular health benefits and that blackcurrant anthocyanins particularly could be a safe and promising supplement to use alongside conventional treatments for patients affected by open-angle glaucoma and other ophthalmic disorders.

Recently, some authors have focused their research on anthocyanins' role in diabetic retinopathy and age-related macular degeneration (Nabavi *et al*., 2015; Sin *et al*., 2013). The authors highlighted that anthocyanins may exert a positive effect on these pathologies owing to their antioxidant activity and other biochemical actions markedly reducing the vision loss and retinal degeneration. However, the evidences are still lacking, and to better clarify the role of anthocyanins in the evolution of diabetic retinopathy and age-related macular degeneration, further studies are needed.

Metabolic disorders

Fruit and vegetables are sources of many antioxidant molecules and may reduce the risk of insurgence of metabolic disorders, such as obesity and type 2 diabetes associated with insulin resistance (Anderson *et al*., 2004), by regulating blood glucose levels or inducing insulin production through pancreatic β-cells in type 2 diabetes (Ghosh and Konishi, 2007a, 2007b). Anthocyanins play a predominant role among dietary bioactive compounds, as demonstrated for the first time by Tsuda *et al*. (2003) when they observed significantly reduced body fat accumulation and plasma glucose concentration induced by high-fat meals. These observations were ascribed to the ability of these molecules to interact with adiponectin, one of the most important adipocytokines that suppresses lipid synthesis in the liver and in white adipose tissue and increases insulin sensitivity in human adipocytes (Tsuda, 2008). Consumption of anthocyanin-rich foods was also associated with a lower risk of type 2 diabetes, while no significant correlations were found for other flavonoid subclasses. Among anthocyanins, Cy and Cy-3-Glu were found to induce some lipid metabolism-related genes and to modulate the gene expression of adipocytokines in human adipocytes (Tsuda *et al*., 2006). *In vitro* evaluation of the gene expression profile in isolated rat adipocytes treated for 24 h with these anthocyanins at 100 nM concentration led to the identification of 633 and 427 upregulated genes, including lipid metabolism and signal transduction-related genes (Tsuda *et al*., 2005). Other anthocyanins have also been tested for potential antidiabetic activity. In a study using male streptozotocin-induced diabetic Wistar rats, intraperitoneal injection of Pg was found to normalize elevated glycaemia and improve serum insulin levels in diabetic rats, shown by serum levels of superoxide dismutase, catalase, malondialdehyde and fructosamine reverting to physiologic values (Roy *et al*., 2008). In a type 2 diabetes mutant mouse (KK-Ay) model, anthocyanin intake, particularly Cy-3-Glu, was found to inhibit elevation of blood glucose levels and to improve insulin sensitivity by upregulating glucose transporter 4 (Glut4) expression with the consequent downregulation of retinol-binding protein 4 (Tsuda *et al*., 2003; Sasaki *et al*., 2007). This triggered an inhibitory effect on the reduction of insulin sensitivity in peripheral tissue and on glucose release following excessive gluconeogenesis.

However, this antidiabetic effect was not observed for bilberry extract, probably owing to the low concentration of Cy-3-Glu. Nevertheless, bilberry extract intake activates adenosine monophosphate-activated protein kinase pathways in white adipose tissue and skeletal muscle, inducing glucose transporter 4 upregulation and consequently enhancing glucose uptake by tissues. In the liver, however, this extract reduces glucose production by efficiently ameliorating hyperglycemia status in type 2 diabetic mice and their liver and serum lipid content via upregulation of peroxisome proliferator-activated receptors α and acylCoA oxidase (Takikawa *et al*., 2010). In addition to the aforementioned retinol-binding protein 4 and adenosine monophosphate-activated protein kinase pathways, it has been suggested that anthocyanins may counteract diabetes via inhibition of α-glucosidase activity in the small intestinal endothelium, delaying the liberation of glucose from dietary complex carbohydrates and, at the same time, glucose absorption, thereby reducing the postprandial glycemic peak. This inhibitory effect of anthocyanins on α-glucosidase activity varies greatly and depends on their molecular structure; anthocyanin glycosides were found to be very weak enzyme inhibitors (Iwai *et al*., 2006; Kumar *et al*., 2011), while acylated anthocyanins were found to have the best inhibitory activity (Matsui *et al*., 2001, 2002) owing not to anthocyanidin itself but to the caffeoyl sophorose component of the acylated anthocyanin molecule (Matsui *et al*., 2004).

Two further *in vivo* studies provide evidence to support the anti-obesity effect of anthocyanins on high-fat diets, showing a significant reduction of serum triglyceride and cholesterol levels and a marked increase in high-density lipoprotein cholesterol concentration (Pojer *et al*., 2013; Kianbakht *et al*., 2014; Asgary *et al*., 2014). Although several studies have been conducted on anthocyanins and their effects on metabolic disorders, some results still appear controversial. Sometimes, this is because of a different anthocyanin composition of the food or preparation being used or to differences in experimental conditions such as dietary contribution and dosage of test samples. Furthermore, extensive *in vivo* studies and clinical evaluations to validate antidiabetic and anti-obesity effects are still lacking.

Cardiovascular diseases

Cardiovascular disorders, the biggest cause of death worldwide, are a class of diseases that affect the heart and/or blood vessels, and disease progression is closely linked to platelet aggregation, hypertension, high plasma concentration of LDL cholesterol and vascular endothelium dysfunction. Indeed, while the term technically refers to any disease that affects the cardiovascular system, it is often used as an umbrella term to include other related pathologies like atherosclerosis and/or hypertension (Aviram *et al*., 2005; Serban *et al*., 2015). Dietary antioxidants, including anthocyanins, seem to have a potential role in preventing or partially reverting these pathological conditions. Many studies have been carried out in this area (Wallace *et al*., 2016; Wang *et al*., 2014).

Here, we will first review those that have examined the properties of purified anthocyanins and then those concerning the properties of anthocyanin-rich foods, some of which are shown in Table 2.

According to the findings of Hassellund *et al*. (2013), purified anthocyanin supplements ameliorate cardiovascular metabolic risk factors and markers of inflammation and oxidative stress in prehypertensive and non-dyslipidemic participants, increasing plasma polyphenol concentration 1–3 h post-ingestion. This increased bioavailability resulted in a significant rise in high-density lipoprotein cholesterol and blood glucose after anthocyanin ingestion compared with a placebo.

Many studies have investigated the properties of anthocyanins to counteract peroxynitrite-induced endothelial dysfunction and vascular failure. Cy-3-Glu was found to act (Serraino *et al*., 2003) as an efficacious scavenger of peroxynitrite radicals, but its ability did not only seem to be limited to its antioxidant power but also to be dependent on the regulation of enzymes involved in nitric oxide (NO) synthesis; this claim was confirmed by a significant decrease in the expression levels of inducible NO synthase. This was confirmed by Sorrenti *et al*. (2007) in a study on human endothelial cells in which the authors also demonstrated an additional cytoprotective effect of Cy-3-Glu owing to the induction of the stress protein heme oxygenase-1, an important mediator in the cellular protective mechanism against oxidative injury. Recently Chen *et al*. (2011) investigated the effect of Dp on monocyte adhesion to endothelial cells induced by oxidized LDL (ox-LDL). The results showed that pretreatment with Dp (concentration range 50–200 μM) dose-dependently decreased the ox-LDL-induced upregulation of intercellular adhesion molecule 1 and P-selectin expression, enhancing both adhesion and transmigration of monocytes. Moreover, Dp was observed to be involved in many other ox-LDL-induced mechanisms in endothelial cells in a dose-dependent manner, including ROS generation, p38 mitogen-activated protein kinase (MAPK) protein expression, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) expression and transcription activity, IkB-α degradation, dihydro nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mRNA expression.

Anthocyanins would also seem to exert their cardiovascular protective effects through antiinflammation and antiplatelet activities (Lucioli, 2012). Garcia-Alonso *et al.* (2009) showed that anthocyanins significantly inhibit TNFα- induced inflammation through monocyte chemoattractant protein-1 in the human endothelium. Dp, in particular, suppresses myocardial ischemia-reperfusion injury through inhibition of signal transducers and activators of transcription 1 activity in cardiac muscle (Scarabelli *et al*., 2009), while Cy-3-O-β-Glu is able to inhibit inflammation by slowing atherosclerosis progression, although this effect appears to be ascribable to its metabolite, protocatechuic acid (Wang *et al*., 2011). However, other mechanisms including inhibition of lipoprotein oxidation, free radical scavenging activity and modulation of eicosanoid metabolism seem to be implicated in the reduction of atherosclerosis (Mauray *et al*., 2012; Wallace *et al*., 2016) as shown by Tsuda *et al*. (1996) in a UV light radiation-induced lipid peroxidation model in which three purified anthocyanins (Pg-3-Glu, Cy-3-Glu and Dp-3-Glu), as well as their aglycones, were found to be involved in the marked inhibition of lipid peroxidation by acting as active oxygen radical scavenging agents.

Researchers have long wondered, however, what the effects of these phytochemicals are when consumed as anthocyanin-rich foods.

Following the pioneering studies on red wines in which it was demonstrated that daily consumption markedly raised serum total antioxidant capacity and decreased LDL oxidation compared with the baseline (Cao *et al*., 1998), many epidemiological studies have been carried out with results suggesting that consumption of red wine may have cardioprotective effects (Strandberg *et al*., 2007; Streppel *et al*., 2009; Hansen-Krone *et al*., 2011; Levantesi *et al*., 2013). Nevertheless, when compared with dealcoholized wine or red grape juice and normalized for polyphenol content, the beneficial effects of red wine are attributable entirely to polyphenols, particularly to anthocyanins. Moreover, red grape juice was found to be much more effective than red wine or dealcoholized red wine at the same polyphenol dose in inhibiting atherosclerosis and improving lipids and antioxidant parameters (Vinson *et al*., 2001; Erlund *et al*., 2008). Other anthocyanin-rich foods have also been extensively studied, as can be seen from the *in vitro* and *in vivo* studies reported in Table 2.

Cyanidin-3-glucoside from blackberry extract showed antiinflammatory activity in J774 cells due, at least in part, to the suppression of NO production through the same mechanisms and signalling pathways described previously for the purified compound (Pergola *et al*., 2006). However, a further two plausible mechanisms of action were observed: Cy-3-Glu was found to enhance eNOS expression and NO production via the proto-oncogene tyrosine-protein kinase (Src)-extracellular signal-regulated kinase 1/2 (ERK1/2)- Sp1 Transcription Factor (Sp1) signalling pathway (Xu *et al*., 2004a) and eNOS activity by regulating its phosphorylation (Xu *et al*., 2004b) in an *in vitro* bovine artery endothelial cell model.

The Kuopio ischemic heart disease risk factor study demonstrated that subjects who consumed a large amount of berries rich in anthocyanins had a significantly lower risk of cardiovascular disease (CVD)-related death than those in the low-consumption group (Rissanen *et al*., 2003). A statistically significant correlation between strawberry consumption and mortality due to CVD was also revealed in the Iowa women's health study involving postmenopausal women (Mink *et al*., 2007) and in other human trials (Erlund *et al*., 2008; Mink *et al*., 2007; Gupta *et al*., 2009) in which consumption of anthocyanin-rich foods like blackcurrants, bilberries and blueberries was related to reduced LDL cholesterol levels, increased plasma antioxidant capacity and inhibitory effects on atherosclerosis (Ellingsen *et al*., 2008). The effects of raspberry, strawberry and bilberry juices associated with an atherogenic diet have also been investigated. After 12 weeks, it was found that berry juices inhibited aortic lipid deposition by approximately 90% and reduced hepatic antioxidant enzyme activity without any lowering of plasma cholesterol levels (Yamanouchi *et al*., 2000).

In addition to decreasing oxidative stress in the vascular endothelium, another extremely important aspect related to CVDs has been studied. According to the results obtained by Ziberna *et al*. (2012) from their investigation into the acute direct effects of bilberry anthocyanins on whole rat hearts under ischemia-reperfusion conditions, anthocyanins exert cardioprotective effects. The results showed that perfusion with a low concentration of bilberry anthocyanins (0.01–1 mg/L) significantly decreased ischemia-reperfusion injury: inhibiting lactase dehydrogenase release, increasing post-ischemic coronary flow and decreasing the incidence and duration of reperfusion arrhythmias. In contrast, a high concentration of anthocyanins (5–50 mg/L) was found to be cardiotoxic through a pro-oxidant mechanism (Halliwell, 2008). Under reperfusion conditions, anaerobic metabolism may lead to a decrease in intracellular pH values; this event may enhance the radical scavenging properties of anthocyanins (Cvorovic *et al*., 2010) that are known to be related to the molecular structure of anthocyanins. All these features allow us to say with reasonable certainty that despite low bioavailability, anthocyanins may have significant cardioprotective properties.

Finally, we should add that the cardioprotective activity observed is not only due solely to the primordial molecules but is also attributable to anthocyanin metabolites (Tsuda, 2012) as described earlier for colonic metabolite protocatechuic acid, which slows atherosclerosis progression by inhibiting the inflammation process (Pojer *et al*., 2013).

Antimicrobial activity

The important role of phenolic compounds in defence against beneficial and pathogenic human intestinal bacteria has been extensively investigated (Nohynek *et al*., 2006). Some anthocyanins tested (including Pg, Cy and Dp as well as Cy-3-Glu) were found to be effective inhibitors of the Gram-negative *Escherichia coli* strain CM 871 (DNA repair deficient). However, the same behaviour was not found for wild-type *E. coli* and beneficial Gram-positive bacteria. The anthocyanin fraction was found to be the most active of berry polyphenol compounds in reducing viability of *Salmonella entericaserovar Typhimurium* (Nohynek *et al*., 2006); this fraction, in fact, tended to induce lipopolysaccharide release from the outer membrane of Gram-negative bacteria. Bilberry and blueberry extracts showed inhibitory effects on the growth of a wide range of Gram-positive and Gram-negative bacteria, where *Citrobacter freundii* and *Enterococcus faecalis* emerged as the most sensitive strains while *E. coli* showed the greatest resistance (Burdulis *et al*., 2009; Wu *et al*., 2008; Cesoniene *et al*., 2009) as described earlier for some pure anthocyanins. However, this ability would seem to be closely related to neutral pH conditions, as the anthocyanins probably showed reduced antimicrobial activity due to their loss of chemical stability (Lacombe *et al*., 2010). Some food-derived products rich in anthocyanins were also found to inhibit the growth of *Staphylococcus aureus* and *Enterococcus faecium* strains, while only weak effects were observed on *E. coli* and *Saccharomyces cerevisiae* (Werlein *et al*., 2005). There are different mechanisms to explain the antimicrobial activity of anthocyanins. Not only can they cause structural damage to bacterial cells thereby destroying cell wall integrity and destabilizing the cytoplasmic membrane and intracellular matrix leading to membrane condensation of cellular material (Cisowska *et al*., 2011) but anthocyanins can also inhibit extracellular microbial enzymes (Naz *et al*., 2007) by directly affecting the microbial metabolism that leads to a deprivation of the substrates required for microbial growth (Burdulis *et al*., 2009).

As described earlier, microbial strains have different susceptibilities to berry extracts, with cloud berries showing the greatest antimicrobial activity followed by raspberries and strawberries (Nohynek *et al*., 2006) while no effect on the growth of yeast species was observed. Berry extracts have also shown interesting bactericidal activities against a Gram-negative bacterium *Helicobacter pylori*; all extracts analysed showed >70% inhibition (cranberry, elderberry and bilberry), and blueberry showed the best inhibitory activity (>90%) (Chatterjee *et al.*, 2004). Nevertheless, it is known that anthocyanins positively modulate the intestinal bacterial population by enhancing the growth of *Bifidobacterium spp*. and *Lactobacillus-Enterococcus spp.*, so the results appear controversial in attributing the final antimicrobial effects observed directly to the anthocyanin compounds (Pojer *et al*., 2013).

Brain health

Anthocyanin-rich foods may also have beneficial effects against cognitive decline and age-related neurodegeneration and seem able to modulate neuronal functions (Tsuda, 2012; Rendeiro *et al*., 2012). Some studies investigated different food-derived anthocyanins in animal models. One of these conducted by Shukitt-Hale *et al.* (2009) observed that blackberry and plum anthocyanin extracts were able to delay the onset of neural function decline and to improve both cognitive and motor function by inhibiting neuro-inflammation and modulating neural signalling. Nevertheless, other plausible mechanisms may be involved that account for the benefits described earlier, such as cerebral blood flow improvement (Spencer, 2010) and inhibition of NF-kβ upregulation (Goyarzu *et al*., 2004). Shukitt-Hale *et al.* (2008) previously demonstrated, by highlighting the suppression of IL-1b, TNF-α and NF-kβ expression in the hippocampus, that blueberry intake inhibited the cognitive and motor function impairments induced by kainic acid. The mechanism through which berries exert their positive effects against age-related cognitive decline and neurodegeneration seems to be due to an increase of dopamine release in the brain, which prompts neurons to enhance intracellular communication (Shukitt-Hale *et al*., 2009). Bilberry-fed animals also showed an improvement in memory, vision and sensory input control (Galli *et al*., 2006), while lyophilized berry-fed rats showed enhanced short-term memory and improved working memory (Ramirez *et al*., 2005). Rendeiro *et al.* (2012) further suggested that blueberries can improve memory and learning in elderly animals by modulating important structural and synaptic plasticity markers, as previously observed by Joseph *et al.* (2003). The authors in fact found a positive correlation between a blueberry-rich diet and improved neuronal function, highlighting delayed development of Alzheimer's disease, a decrease in spatial memory deficits and an enhancement of memory-associated neuronal signalling as well as influencing synaptic plasticity regulation and the consolidation of learning and memory (Joseph *et al*., 2003). These features together with the observation that blueberry polyphenols may improve memory by acting on the hippocampus and stimulating neurogenesis (Shukitt-Hale *et al*., 2008) have been confirmed by a human clinical study (Table 2) in which male subjects who consumed blueberry juice for 12 weeks showed improved memory performance (Krikorian *et al*., 2010).

Antiinflammatory activity

Inflammation is a complex biological response to tissue injury and is associated, providing there is a favourable microenvironment, with the onset, development and progression of cancers or tumours (Coussens and Werb, 2002). Inflammation events are closely related to cyclooxygenase (COX) enzyme activity that converts arachidonic acid to proinflammatory cytokines, the prostaglandins. Therefore, to exert effective antiinflammatory activity, an inhibitory effect on COX enzymes is highly desirable (Seeram *et al*., 2001). The first study to highlight Cy as the best antiinflammatory showed decreases in COX-1 and COX-2 activities by 52% and 74%, respectively (Seeram *et al*., 2001), with respect to the positive control (aspirin) in the COX activity assays. Since then, other studies have evaluated purified anthocyanin fractions obtained from different anthocyanin-rich foods (cherries, bilberries, blueberries, cranberries, etc.) (Seeram *et al*., 2001). All anthocyanin fractions studied demonstrated an inhibitory effect on both COX-1 and COX-2 enzymes with strawberry, blackberry and raspberry fractions showing the highest activity, comparable with that of the positive controls, ibuprofen and naproxen (Seeram *et al*., 2001). Like Cy, Dp was shown to inhibit COX-2 expression, unlike Pg, Pn and Mv.

In addition, Dp is known to suppress the activation of MAPK, which is directly involved in cellular responses to a wide range of stimuli such as mitogens and proinflammatory cytokines (Hou *et al*., 2005). This property seems to belong only to anthocyanins characterized by an ortho-dihydroxyphenyl structure (Hou *et al*., 2005).

As observed in many *in vitro* studies, anthocyanins have the ability to inhibit mRNA and/or protein expression levels of COX-2, NF-kβ and various interleukins. Moreover, in several cell-based models, it seems that anthocyanins and their aglycones could also inhibit human prostaglandin synthase activity (Afaq *et al*., 2005; Boivin *et al*., 2007). These observations have also been confirmed by an *in vivo* study on rats with carrageenan-induced lung inflammation treated with blueberry anthocyanins (Cy-3-O-Glu 80% titrated), which found a significant dose-dependent reduction of all inflammation parameters considered (Rossi *et al*., 2003). In humans, red wine-derived anthocyanins seem to inhibit TNF-α-induced inflammation through modulation of endothelial monocyte chemoattractant protein-1 as described earlier for the mechanisms underlying the cardioprotective properties of anthocyanins (Garcia-Alonso *et al*., 2009).

Recently, the effects of Cy-3-O-Glu on synthetic and metabolic activity of ethanol-stimulated human pancreatic stellate cells were evaluated by Cesna *et al.* (2015). The authors highlighted the ability of this compound to inhibit proliferation of activated human pancreatic stellate cells and synthesis of fibronectin and collagen I reverting their metabolic activity. This observation allows to hypothesize the use of anthocyanins as antifibrogenic agents in treatment and/or prevention of pancreatic fibrosis, although further studies are needed to

clarify the mechanism of action underlying this important antiinflammatory effect.

Anticarcinogenic activity

The anticancer properties of anthocyanins have been largely based on evidence from *in vitro* cell-based assays (He and Giusti, 2010) and may be due to multiple and sometimes additive mechanisms. These events include: cell cycle arrest (G1/G0 and G2/M) (Renis *et al.*, 2008), apoptosis (Yi *et al*., 2005) and anti-angiogenesis (Bagchi *et al*., 2004), as well as inhibition of DNA oxidative damage (Singletary *et al.*, 2007), induction of detoxification phase II enzymes (Srivastava *et al.* 2007), anti-mutagenic effects (Ohara *et al.*, 2004, Yoshimoto *et al.* 2001) and anti-carcinogenic effects (Shih *et al.*, 2007) and the previously mentioned inhibition of COX-2 enzymes. The anti-proliferative activity of anthocyanins occurs in different cell types (Zhao *et al*., 2004; Zhang *et al*., 2006; Yi *et al*., 2005). They block various cell cycle stages that influence regulatory proteins as well as selectively inhibiting cancer cell growth. Recently, He and Giusti (2010) reviewed some *in vitro* cell-based assays, concluding that plant or food-derived anthocyanins and anthocyanidins were found to be more potent than combined non-anthocyanin flavonoids as regards cell growth inhibition in a human malignant intestinal carcinoma-derived cell line (HTC-15) and a human gastric cancer-derived cell line (AGS), as well as in two human colon cancer-derived cell lines (HT-29 and Caco-2). Some anthocyanin-rich extracts have also been found to be able to inhibit the growth of human malignant HT-29 colon cancer cells but not that of nonmalignant colon-derived NCM460 cells, although the mechanism involved in this selective effect is not known (Zhao *et al*., 2004). It has been hypothesized that anthocyanins may act not only through intrinsic pathways, increasing mitochondrial membrane potential, cytochrome c release and modulating pro-apoptotic proteins (Chang *et al*., 2005), but also via extrinsic pathways, modulating expression of FAS receptors and FAS ligands in cancer cells leading to apoptosis (Feng *et al*., 2007). Anthocyanins were also found to be able to inhibit the invasion of multiple cancer cell types, reducing the expression of matrix metalloproteinase and stimulating inhibitors of the latter (Brandstetter *et al*., 2001). These compounds also appear able to suppress angiogenesis events by inhibiting vascular endothelial growth factors expression, which plays an important role in tumour vascularization (Bagchi *et al*., 2004; Favot *et al*., 2003; Huang *et al*., 2006).

Highly purified anthocyanins have also been evaluated. Four strawberry-derived anthocyanins at concentrations of 100 μg/mL have been shown to reduce cell viability of human oral (CAL-27, KB), colon (HT-29, HCT-116) and prostate (LNCaP, DU145) cancer cells, although the different sensitivity levels detected were probably due to the different chemical structure of each compound analysed, including aglycone type, glycosylation pattern and acylation (Zhang *et al*., 2006; Jing *et al*., 2008). Two exploratory clinical studies highlighted an increasing prostate-specific antigen doubling time in patients with prostate cancer after pomegranate preparations intake (Pantuck *et al*., 2006; Paller *et al*., 2013). However, recent studies did not confirm this observation in light of the high difference between pomegranate preparations in terms of active principal content (Chrubasik-Hausmann *et al*., 2014). The authors conclude that, before the beginning of the study, in order to avoid a wrong conclusion and guarantee the safety and efficacy, the first step should focus on an appropriate standardization of preparations and to adopt all necessary features to avoid a loss of active ingredients during storage (Chrubasik-Hausmann *et al*., 2014; Vlachojannis *et al*., 2015a, 2015b).

Anthocyanidins have been demonstrated to be better inhibitors of cell proliferation than anthocyanins (Zhang *et al*., 2006), with Dp having the best growth inhibition property owing to the presence of hydroxyl groups on the B ring of the anthocyanidin core, which seems to facilitate this molecule exerting its effect by blocking activation of the MAPK pathway (Hou, 2003). Cy and Dp have also shown growth inhibition properties via inactivation of the glutathione antioxidant system and promotion of oxidative stress, while Pg and Mv have not shown any such effect (Cvorovic *et al*., 2010). These findings are reported in two studies conducted by Hou *et al*. (2003) on human leukemia cells (HL-60) and in JB6 mouse epidermal cells model, respectively. In the first, the authors concluded that Dp, Pt and Cy induced apoptosis and DNA fragmentation, while Pg, Pn and Mv showed no induction of apoptosis. Once again, the highest anticancer activity was shown by Dp and involved gene expression and activation of caspase-3 (pro-apoptotic gene) and activation of oxidative stress-induced signalling pathways (Hou, 2003). In the second study, the authors showed 12-O-tetradecanoylphorbol-13-acetate-induced cell transformation and activator protein-1 (AP-1) transactivation to be inhibited by Dp, Pt and Cy but not by Pg, Pn and Mv, suggesting that the ortho-dihydroxyphenyl structure on the B-ring may be essential. Again, Dp had the strongest inhibitory effect on activator protein 1 activation via inhibition of the MAPK signalling pathway and through a synergistic effect with superoxide dismutase enzyme (Hou *et al*., 2003).

The metabolites of anthocyanins also seem to have anticancer properties, as demonstrated by Forester and Waterhouse (2008), who observed that some anthocyanin metabolites from Cabernet Sauvignon grapes showed limited toxicity in Caco-2 cells, showing instead clearly suppressed cell proliferation and induced apoptosis at 140 μM. This behaviour is probably ascribable to their pro-oxidant activity due to the release of hydrogen peroxide, which would oxidize any enzymes present (Forester and Waterhouse, 2008).

In light of the aforementioned, anthocyanins seem to be responsible, at least in part, for the anticancer properties of many fruits and vegetables, but some studies have shown that, in effect, they work collaboratively with other phytochemicals to exert their anticarcinogenic effects. Seeram *et al*. (2001) have demonstrated, for example, that both anthocyanin and proanthocyanidin fractions isolated from cranberry extract exhibited a substantial anti-proliferative effect on all human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1, RWPE-2, 22Rv1) cancer cell lines studied, yielding better results than those from total cranberry extract or flavonol glycosides and organic acid fractions. However, the highest activity was achieved by using a combination of anthocyanins and proanthocyanidins. This was also confirmed by Jing *et al*. (2008), who suggested combining mainly

additive rather than synergistic or antagonistic effects of food-derived anthocyanins and other polyphenols.

The chemopreventive effects of anthocyanins have also been investigated in many animal studies related primarily to gastrointestinal tract-related organs and colon (He and Giusti, 2010), probably due to the high availability of these compounds at this level (He *et al*., 2009).

Bruce *et al*. (2000) suggested that colon cancer development may involve two mechanisms: a localized irritation that produces a local inflammatory response and an electrolyte imbalance. Both mechanisms result from a defect in the epithelial barrier, and both lead to elevated ROS and COX-2 levels in epithelial cells. Anthocyanins, being powerful antioxidants and COX-2 inhibitors, are therefore able to reverse these conditions. However, Lala *et al*. (2006) suggested that the inhibitory effect of dietary anthocyanins in a rat colon cancer model was primarily attributable to the direct effect of improving colon luminal conditions, promoting fecal moisture content and fecal excretion of bile acids (recognized endogenous tumour-promoting compounds) and by protecting the epithelial cells against oxidative damage and microbial infection.

The anticancer activity of anthocyanins has been detected in different animal models. For example, in ApcMin intestinal cancer mouse models fed with cherry extract anthocyanins, the authors highlighted a 74% decrease of cecal tumours in the treated animals with respect to the control group (Kang *et al*., 2003). A few years later, Cooke *et al*. (2005) observed in the same animal model fed with Cy-3-glc or bilberry anthocyanins a decrease in adenoma size by 45% and 30%, respectively, with respect to the control group. Rat colon cancer models fed with bilberry, chokeberry or grape anthocyanins showed aberrant crypt foci reduced by 26% to 29%, achieved by decreasing cell proliferation and COX-2 gene expression (Lala *et al*., 2006). Similar results were obtained using commercially available anthocyanin-rich extract (Magnuson *et al*., 2003). Despite these encouraging results, we must not forget that the anticancer properties of anthocyanins are related to their bioavailability (He *et al*., 2009). A number of research groups have attempted to identify the plasma concentration range that may exert this beneficial effect.

Many *in vitro* studies, some of which are discussed earlier, have shown that anthocyanins exert their beneficial effects in a concentration range of 10^{-6} to 10^{-4} M. However, some human studies on anthocyanin uptake have suggested that they reach levels of 10^{-8} to 10^{-7} M in human blood, concentrations far below those required to exhibit *in vitro* anticarcinogenic effects. Thus, it is not clear whether the *in vivo* concentrations are sufficient to induce anticancer effects on humans or if anthocyanins exert chemopreventive effects by themselves or must be hydrolysed to aglycon forms to exert their properties (Cooke *et al*., 2005). These properties have been extensively reviewed by various authors (Wang and Stoner, 2008a, 2008b; Thomasset *et al*., 2009), but to date, results are still controversial, probably owing to inadequate human studies, which often have failed to detect any significant association between anthocyanidin intake and anticancer properties (Wang and Stoner, 2008a, 2008b; Rossi *et al*., 2007; Bosetti *et al*., 2006).

DRUG INTERACTIONS

All dietary polyphenols are extensively metabolized in humans through drug-metabolizing enzymes. Many flavonoids, for example, have been found to be able to increase expression of drug-metabolizing enzymes via several signal transduction pathways, to compete with drugs or other xenobiotics as enzymatic substrates (i.e. drugs or other xenobiotics) and to inhibit these enzymes in a non-competitive manner. Although several reviews focused on flavones, flavonols, flavanones, flavanols and/or isoflavones are currently available, the same does not apply to anthocyanins, despite the fact that these compounds are recognized to be the most abundant flavonoid constituents in plant, foods and in several dietary supplements (Wang and Stoner, 2008a, 2008b).

The drug-metabolizing enzymes (especially those of Phase I) belonging primarily to the cytochrome P450 (CYP) superfamily play a crucial role in the metabolism of drugs and other xenobiotic compounds because they are responsible for the biotransformation of active drugs into their active or inactive metabolite(s) and, sometimes, for the bioactivation of xenobiotics (Gonzalez and Tukey, 2006; Xu *et al*., 2005). In light of this, the modulation of these enzymes may have serious pharmacological and toxicological consequences. Some studies have analysed the effects of anthocyanins and anthocyanidins on drug-metabolizing enzymes belonging to the CYP superfamily, in particular, the isoform 3A4 (CYP3A4), which is responsible for the metabolism of more than 50% of clinical pharmaceuticals (Kimura *et al*., 2010). It would seem that anthocyanins cause direct inhibition of human CYP3A4, in a concentration-dependent manner, as demonstrated by Dreiseitel *et al*. (2008), or affect their expression by interacting with several regulatory pathways (Rodríguez-Fragoso *et al*., 2011). Anthocyanins and anthocyanidins have been found to be weak inhibitors of the enzymatic isoform 3A4 with IC_{50} values of 12.2–46.5 μM and 74.1 to 249 μM, respectively, especially when compared with other polyphenols like naringenin, quercetin and curcumin (Dreiseitel *et al*., 2008). Nevertheless, they were found to be superior to phenolic acids, which showed only a negligible inhibition with IC_{50} values ranging from 472.3 to 7842 μM. Another *in vitro* study that investigated the modulatory effects of ten anthocyanins and six anthocyanidins on human CYP2C19 highlighted that the tested compounds were able to inhibit this enzymatic isoform in a concentration-dependent manner as follows: anthocyanidins (IC_{50} values 20.2 to 62.9 μM) > anthocyanidin-monoglucosides (IC_{50} values 138.0 to 266.7 μM) > anthocyanidin-diglucosides (IC_{50} values >316 μM) (Sand *et al*., 2010). These results revealed glycosylation to be a key factor in granting inhibitory potential to anthocyanins, in line with results also obtained with CYP3A4.

Some studies have also analysed anthocyanin-rich food-derived products. Cranberry and other red fruit or vegetable juices containing anthocyanins were shown to cause inhibitory effects on CYP3A4 in human liver and rat intestinal microsomes (Ngo *et al*., 2009; Kim *et al*., 2006). These observations were also confirmed by an *in vivo* study (Uesawa and Mohri, 2006). Cranberry juice was found to diminish CYP2C9 activity versus diclofenac and phenytoin in human liver microsomes, although this occurrence was not observed

clinically (Ushijima *et al*., 2009a; Ushijima *et al*., 2009b). In addition to the isoforms 3A4 and 2C9, anthocyanins and their aglycones were found to possess weak inhibitory activity on CYP2D6 although 1000-fold lower with respect to grapefruit-derived furanocoumarins; on the basis of this observation, interference with drug metabolism by CYP2D6 is highly unlikely (Dreiseitel *et al*., 2009).

In phase II biotransformation, anthocyanidins undergo extensive glucuronidation (Milbury and Kalt, 2010), behaving like UDP-glucuronyl transferase enzyme substrates and being able to inhibit other enzymes like sulfotransferase and glutathione S-transferase. These compounds compete with other drugs, xenobiotics or their biotransformation products, thereby decreasing their metabolism. Therefore, anthocyanin–drug interactions may be very common at this level (Bartikova *et al*., 2013). Despite evidence to date regarding significant changes in drug pharmacokinetics after anthocyanin intake, the literature is still lacking, and these possibilities deserve more attention.

SAFETY AND TOXICOLOGICAL ASPECTS

Anthocyanins are the most widely consumed flavonoid constituents that occur ubiquitously in plants, fruit and vegetables. Their intake has been estimated to be up to ninefold higher with respect to other dietary flavonoids (He and Giusti, 2010). Despite this, their consumption has not been associated with adverse health effects (Pojer *et al*., 2013). The use of anthocyanins from natural sources as colourants in foods and beverages is widely permitted within Europe (E163), Japan, the USA and many other countries having been evaluated by the Scientific Committee on Food in 1975 and the Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives (JECFA) in 1982 (EFSA, 2013). However, European Union (EU) specifications do not indicate which fruits or vegetables can be used to obtain the food additive E163, nor are the extraction or characterization methods described (EFSA, 2013). The latter is an issue that deserves some attention because anthocyanin intake through food colourants was found to be higher (2.7 to 7.8 mg/kg bw/day) than estimated intake from the regular diet (1 mg/kg bw/day for adults and 2 mg/kg bw/day for children) (EFSA, 2013). The acute oral toxicity of these substances has been evaluated mainly using aqueous fruit extracts containing several anthocyanins and has been assessed in mice, rats and rabbits. LD_{50} values were found to be 25 g/kg bw in mice and 20 g/kg bw in rats. No adverse effect was observed on blood pressure in rabbits (Pourrat *et al.*, 1967).

The JECFA (1982) described some short-term studies with anthocyanins, some of these lacking any specifications of the exact composition of the tested extracts. No adverse effects in survival, growth, haematology parameters or histopathology were reported when guinea pigs and weanling Wistar rats were treated with alcoholic extract of fermented blueberries juice in the presence of yeasts at doses of 3 g/day for 15 days and 0, 1, 2 or 3 g/day for 90 days, respectively (Pourrat *et al.*, 1967).

No statistically significant differences in body weight, growth, survival, clinical parameters, organ weight or pathological lesions were noted between beagle dogs treated with 0, 7.5 or 15% grape skin extracts (approximately 2.4% anthocyanins by weight) for 90 days with respect to the control group (Cox and Babish, 1978). Likewise, no biologically relevant effects on mortality, body weight, ophthalmic examination, haematology or clinical parameters were observed in Sprague-Dawley rats fed with diets containing grape seed (0.63, 1.25 and 2.5% (w/w) of diet) or grape skin extracts (2.5% (w/w) of diet) for 90 days. The same extracts did not show any teratogenic or mutagenic effects. However, data regarding long-term toxicity and carcinogenesis of anthocyanins are still lacking. A two-generation reproduction study using a grape skin extract preparation containing approximately 3% of anthocyanins was performed in Sprague-Dawley rats fed with dietary levels of 7.5% and 15% of the grape skin extract (JECFA, 1982). No differences in reproductive performance or indices including pup viability were found between the controls and treated groups. At a higher dose, rats from both F1 and F2 pups exhibited lower body weight, while no haematological, urinary or histopathological effects were observed (Cox and Babish, 1978). Furthermore, no developmental toxicity in rats, mice or rabbits was observed after administration of an anthocyanin-rich extract from currants, blueberries and elderberries at doses of 1.5, 3 or 9 g/kg bw/day over three successive generations (Pourrat *et al.*, 1967).

Based on early toxicological studies, some of which are described earlier, including mutagenicity, reproductive toxicity, teratogenicity and acute and short-term toxicity evaluations, the JECFA concluded that anthocyanin-rich extracts had very low toxicity (JECFA, 1982). The no-observed-effect-level (NOEL) for young rats was determined to be approximately 225 mg/kg bw in a two-generation reproduction study. Based on the aforementioned result, the estimated acceptable daily intake (ADI) for humans was estimated to be 2.5 mg/kg bw per day, using the equation of ADI = NOEL/100 (Clifford, 2000). Despite this, the European Food Safety Authority panel was of the opinion that these values could not be used to calculate a margin of safety owing to the unclear anthocyanin composition of the extracts analysed. Furthermore, the European Food Safety Authority concluded that it was not possible to extend the Scientific Committee on Food opinion (SCF, 1975) of the safety of food additives derived from natural sources to anthocyanins extracted from other sources or to pure anthocyanins. It also concluded that the toxicological database available at the time was inadequate to establish a numerical ADI for anthocyanins (EFSA, 2013).

CONCLUSION

This paper describes current knowledge of anthocyanin biosynthesis and dietary sources as well as the ever-increasing evidence of their health-promoting effects, providing a more detailed overview of the pharmacological, drug interactions, safety and toxicological aspects, often overlooked, of this important class of phytochemicals, to give the reader a comprehensive and updated framework.

Recently, many papers have been published about *in vitro* and *in vivo* studies performed, and several

hypotheses have been posited about the molecular mechanisms underlying the health effects observed, as well as theories to link anthocyanins antioxidant capacity to their chemical structure. To date, 61 completed and ongoing clinical trials are available on anthocyanins' bioavailability, pharmacokinetics and health effects; however, the safety and toxicological aspects are still lacking, and further studies are strictly recommended.

Conflict of Interest

The authors have no conflict of interest to declare.

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