Anthocyanins in Cardiovascular Disease[1]

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ABSTRACT

Anthocyanins are a group of abundant and widely consumed flavonoid constituents that occur ubiquitously in the plant kingdom, providing the bright red-orange to blue-violet colors present in many fruit- and vegetable-based food products. Their intake has been estimated to be up to 9-fold higher than that of other dietary flavonoids. Anthocyanins have become increasingly important to the food industry as their use as natural alternatives to artificial colors has become widespread and knowledge of their health-promoting properties has become more evident. Epidemiological studies suggest that increased consumption of anthocyanins lowers the risk of cardiovascular disease (CVD), the most common cause of mortality among men and women. Anthocyanins frequently interact with other phytochemicals, exhibiting synergistic biological effects but making contributions from individual components difficult to decipher. Over the past 2 decades, many peer-reviewed publications have demonstrated that in addition to their noted in vitro antioxidant activity, anthocyanins may regulate different signaling pathways involved in the development of CVD. This review summarizes the latest developments on the bioavailability/bioactivity and CVD preventative activities of anthocyanins, including results from in vitro cell culture and in vivo animal model systems as related to their multiple proposed mechanisms of action. Limited yet promising data from epidemiological studies and human clinical trials are also presented. Future studies aimed at enhancing the absorption of anthocyanins and characterizing their metabolic and/or breakdown products are necessary to ultimately evaluate their use for protection/prevention against the development of CVD. *Adv. Nutr. 2: 1–7, 2011.*

Introduction

In 2004, an estimated 17.1 million people died from cardiovascular disease (CVD),[2] mainly from heart disease (7.2 million) and stroke (5.7 million). This number is expected to increase to 23.6 million people in 2030 (1). According to the WHO, CVD is caused by disorders of the heart and blood vessels and includes coronary heart disease (CHD), cerebrovascular disease, peripheral artery disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism. Atherosclerosis is a chronic inflammatory disease caused by plaque rupture or erosion, which leads to acute formation of platelet-rich thrombi that occlude or partially occlude the arterial lumen and causes CVD clinical events such as myocardial infarction, unstable angina, or cerebrovascular accident (2). Behavioral risk factors such as smoking, lack of physical inactivity, and an unhealthy diet account for ~80% of CVD (1). Behavioral risk factors may promptly lead to intermediate risk factors of developing CVD, including obesity, as well as elevated blood pressure, glucose, and lipid levels (1).

Consumption of fruits and vegetables has been inversely associated with a decreased risk of CVD (3), most likely due to the abundance and variety of bioactive compounds present. As an alternative to pharmaceutical medications, consumption of diets rich in natural bioactive components and their contribution to maintaining or improving cardiovascular health has been a subject of considerable interest to researchers. Dietary flavonoids, a large, ~6000-member group of polyphenols, have emerged as potential candidates to protect against CVD, because epidemiological studies associate regular consumption of flavonoid-rich foods and beverages with a decreased risk of CVD mortality. Many published cohort studies suggest that high intakes of flavonoids may be associated with a decreased risk of CVD; however, others find little to no significant association (4). An analysis of 16 cohort studies revealed that as mean flavonoid intake increased, age-adjusted CHD mortality decreased significantly (5). Recently, a 16-y follow-up study of 34,489 CVD-free postmenopausal women in the Iowa Women's Health Study showed that dietary intakes of certain classes of flavonoids, including flavanones and anthocyanidins and certain foods rich in flavonoids, were associated with a reduced risk of death due to CVD and CHD (6).

Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of flavilium salts and are members of the flavonoid family, possessing a characteristic $C_3 - C_6 - C_3$ carbon structure. Plants typically produce anthocyanins as a protective mechanism against environmental stress factors, including UV light, cold temperatures, and drought (7). The chromophore of 8 conjugated double bonds carrying a positive charge on the heterocyclic oxygen ring is responsible for the intense red-orange to blue-violet color produced by anthocyanins under acidic conditions. Anthocyanins

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[2] Abbreviations used: CHD, coronary heart disease; CVD, cardiovascular disease; eNOS, endothelial nitric oxide synthase; GI, gastrointestinal; MCP-1, monocyte chemotactic protein 1; MMP, matrix metalloproteinase; NOS, NO synthase.
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show a λ_{max} between 465 and 550 nm, as well as significant absorption in the UV range between 270 and 280 nm (8). Over 635 anthocyanins have been identified (9). Six anthocyanidins, cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin, occur ubiquitously in nature, accounting for over 90% of the anthocyanins currently identified (9). Anthocyanidins are rarely found in nature because of their poor stability, whereas glycosylated forms predominate with and/or without additional aromatic and/or aliphatic acid conjugation(s). Anthocyanin-rich extracts are increasingly attractive to the food industry as natural alternatives to synthetic FD&C dyes and lakes.

Daily intake of polyphenols has been estimated to be ~1000 mg/d, which is significant when compared with the estimated daily consumption of other phytonutrients such as carotenoids, vitamin E, and vitamin C (estimated at 5, 12, and 90 mg/d, respectively) (10). The daily estimated intake of anthocyanins is high (estimated at 180 and 215 mg/d) compared with the intake of other dietary flavonoids such as genistein and quercetin (estimated at 20–25 mg/d) (11). Anthocyanins are among the few plant polyphenols that can be detected in the plasma in their native intact forms (glycosides). Until very recently, anthocyanins were thought to have a very low bioavailability, with <1% of the ingested amount reaching the plasma; however, some studies reveal that the bioavailability of these compounds may be underestimated, because the metabolites and breakdown products of anthocyanins have not yet been identified (12).

In this review, recent studies on the CVD preventative activities of anthocyanins, including results from in vitro cell culture and in vivo animal model systems as well as data from human epidemiological studies, are presented. Current knowledge of the bioavailability of anthocyanins as well as their breakdown products and metabolites is also presented. Many in vitro laboratory studies provide insight on the multiple mechanisms by which anthocyanins may help maintain a healthy vascular system. The abundance of in vivo animal and human studies is low; furthermore, the relevance of the high concentrations of anthocyanins used in many in vitro studies as related to the in vivo situation needs to be confirmed.

Current status of knowledge

Bioavailability

The biological activities of anthocyanins are closely linked to their absorption and metabolism. A recent study reiterates that anthocyanins are rapidly absorbed in the stomach and intestine of rats (13). An intense red color was present in the acidic extract of all gastric and small intestinal tissue samples, indicating uptake of anthocyanins into the gastrointestinal (GI) tissues. Anthocyanins in the tissues of the rat stomach were identified by their spectral changes at pH 1.0, 4.5, and 10.0 but could not be quantified by HPLC, because they seem to have bound to an unidentified protein. This may be attributed to nonspecific binding or specific binding to a transporter protein (14). Uptake of black raspberry anthocyanins reached 7.5% of the administered dose in the small intestinal tissue, which is much higher than the reported bioavailability of these compounds based on plasma and urine concentrations (13). This suggests that intact anthocyanins may be taken up into the GI tract tissues efficiently but not transported into the circulation. Transport across the apical membrane using an in vitro epithelial Caco-2 cell model occurred to a much larger extent than further translocation of intact black currant anthocyanins across the basolateral membrane (15). Glycosylation and acylation patterns decrease the bioavailability of an anthocyanin; however, glycosidases present in the GI tract may hydrolyze anthocyanins into anthocyanidins, thereby increasing their biological potential but decreasing their stability. The presence of a glucose moiety compared with a galactose or arabinose on the cyanidin and peonidin anthocyanidins present in cranberry juice seems to make them more bioavailable as a percentage of the delivered dose (16).

Anthocyanins exist in the circulation and urine as intact, methylated, glucuronide derivatives and/or sulfoconjugated forms (17–19), reaching peak plasma concentrations between 1 and 3 h after consumption and depending on the individual compound and the food matrix. The metabolites persist in the urine for up to 24 h and may retain their basic anthocyanin structure (19,20). Pharmacokinetic evidence implies that parent glycosides and glucuronide derivatives are prominent in the bloodstream between 0 and 5 h but become increasingly methylated over time (6–24 h), which suggests that the bioactivity of anthocyanins are likely altered over time as a result of metabolic transformation (21).

Several in vivo studies suggest that the food matrix has a significant effect on the absorption and metabolism of anthocyanins. Ohnishi et al. (22) recovered 5% of administered cranberry juice anthocyanins in the urine of humans (22), whereas other researchers recovered between 1.8 and 2% of strawberry anthocyanins (18,23). A recent study of 15 patients with coronary artery disease showed that the total urinary recovery of administered cranberry juice anthocyanins was variable (between 0.078 and 3.2%) among the participants, which is consistent with other berry anthocyanin bioavailability studies (16). The degree of individual variation in anthocyanin bioavailability may result from differences in xenobiotic metabolism in the GI tract, liver, and other tissues. Human polymorphisms have been reported in the genes for catechol-*O*-methyltransferase, glutathione *S*-transferases, and UDP glucuronosyl transferase (24). The variation of human gut microflora may also play a prominent role in the bioavailability of anthocyanins. Microbiota present in the GI tract may metabolize anthocyanins, producing smaller, more bioavailable end-products. The predominance of the colorless carbinol (75–80%) and chalcone (15–20%) forms of anthocyanins present in the blood and urine at neutral pH levels may give rise to rapid degradation of the compounds into smaller phenolic derivatives. As seen in the stomach, intact anthocyanins, their metabolic forms, and decretory products may escape analytical detection by chemically binding to other components such as proteins present in the bloodstream. Shortcomings such as this can be overcome by using labeled anthocyanins in animal and human studies for identification of all metabolites generated.

Gut microflora have the ability to metabolize anthocyanins; however, the literature in this area is limited. Using a bacterial preparation imitating the normal human microbiota population, it is possible to demonstrate the conversion of larger polyphenols to phenolic acids, which demonstrate similar antiinflammatory effects as the parent compounds (25). Microbial deglycosylation and degradation of 6 anthocyanins were investigated in vitro using HPLC-DAD and GC-MS (26). Anthocyanin glycosides in this study were hydrolyzed into anthocyanidins (aglycons) by the microbiota within 20 min to 2 h of incubation depending on the sugar moiety present (26). Because liberated anthocyanidins are very unstable in a neutral pH environment, degradation of the pigments was experienced within 20 min of incubation. Cy-3-rut was first hydrolyzed into cy-3-glu and then into the cyanidin aglycon, which rapidly degraded into protocatechuic acid (3,4-dihydroxybenzoic acid) as a product of human colonic microflora (26). Porcine gut microflora metabolized anthocyanins in vitro into products such as syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), vanillic acid (3-methoxy-4hydroxybenzoic acid), phloroglucinol aldehyde (2,4,6-

trihydroxybenzoic acid), phloroglucinol acid (2,4,6-trihydroxybenzoic acid), and gallic acid (3,4,5-trihydroxybenzoic acid), depending on the individual anthocyanin (26). Smaller phenolic acids and other anthocyanin metabolites have greater chemical and microbial stability, suggesting that they may play an important role in the noted physiological effects and increase in antioxidant activity observed in many studies (26). It should also be noted that degradation-methylated anthocyanins by the gut microbiota may yield de-methylated products.

Epidemiological data

Epidemiological studies have examined the relationship between foods rich in anthocyanins (such as red wine and several species of berries) and CVD as well as the relationship between total anthocyanin intake and risk of developing CVD. Postmenopausal women (n = 34,489) participating in the Iowa Women's Health Study showed a significant reduction in CVD mortality associated with strawberry intake during a 16-y follow-up period (6). Blueberries also showed a significant decrease in CHD mortality using an age- and energy-adjusted model. A significant reduction in RR was associated with the consumption of strawberries and blueberries at least once per week. This cohort study reported that a mean intake of 0.2 mg/d of anthocyanins was associated with reduced risk of CVD in postmenopausal women (6). Female health professionals enrolled in the Women's Health Study (n = 38,176) showed a borderline significant risk reduction of C-reactive protein (CRP) levels among women consuming higher amounts of strawberries. A decreasing trend for CVD was observed in this study for participants who consumed higher amounts of strawberries (27). Several epidemiological studies have shown that CVD mortality can be decreased by moderate consumption of red wine (28,29). A meta-analysis of wine consumption in relation to CVD risk suggests a consistent dose-response cardiovascular preventative effect (30). Numerous human studies suggest that red wine has more favorable effects on lipid metabolism than white wine (31), possibly due to its increased phytochemical content. The "French Paradox" first drew attention to the CVD protective effects of red wine after epidemiological data collected by the WHO revealed a discord in CVD mortality in a cohort of participants from Toulouse, France, compared with other cohorts from 17 Western countries, including the United States and United Kingdom (32–34). The French cohort had a lower risk of CVD mortality despite a higher consumption of saturated fat (32).

Mechanisms

Mechanistic studies support the beneficial effects of flavonoids, including anthocyanins, on the established biomarkers of CVD risk such as NO, inflammation, and endothelial dysfunction (35–37). Inflammation defined by calor (heat), rubor (redness), and tumor (swelling) plays a major role in the development of CVD. The role of anthocyanins in CVD prevention is strongly linked to protection against oxidative stress. Several mechanisms of action have been proposed to explain the in vivo antiinflammatory actions of flavonoids. Anthocyanin isolates and anthocyanin-rich mixtures of flavonoids may provide protection from DNA cleavage, estrogenic activity (altering the development of hormone-dependent disease symptoms), enzyme inhibition, increased cytokine production (thus regulating immune responses), antiinflammatory activity, lipid peroxidation, decreased capillary permeability and fragility, and membrane strengthening (38–42). The chemical structure (position, number, and types of substitutions) of an anthocyanin plays an important role in the biological activity exerted. Dietary anthocyanins have been shown to accumulate in the tissues of pigs during long-term feeding and have a longer residence time in tissues than in the bloodstream (43). Whether anthocyanins accumulate in the cardiac or vascular tissues during long-term feeding is still unknown; however, data from animal studies have shown that anthocyanins affect vascular reactivity (44). Relatively low-dose anthocyanin interventions with patients clinically diagnosed with vascular diseases have been associated with significant reductions in ischemia (45), blood pressure (46), lipid levels (47), and inflammatory status (48). Commercial grape juice (10 mL/kg) has been shown to significantly inhibit platelet activity and experimental coronary thrombosis in vivo (49). Corn-derived anthocyanins made the myocardium less susceptible to ischemia-reperfusion injury ex vivo and in vivo compared with the anthocyanin-free control (50). Clinical studies show little effect of proinflammatory markers on healthy human participants; however, a recent study by Karlsen et al. (51) showed significant improvement of plasma risk biomarkers after supplementation with anthocyanins (51).

NO. The endothelium regulates vascular homeostasis by producing factors that act locally in the vessel wall and lumen, including NO. NO is a signaling molecule that influences the development of atherosclerosis and many aspects of inflammation, ranging from its own production to immunocompetent cells to the recruitment of leukocytes (52). NO is produced from L-arginine by 3 NO synthase (NOS) enzymes: endothelial NOS (eNOS), neuronal NOS, and inducible NOS. NO is a potent vasodilator with antihypertensive, antithrombotic, antiatherogenic, and antismooth muscle proliferative properties (53). The eNOS protein has been shown to be impaired in conditions associated with atherosclerosis, hypertension, diabetes, and ischemia-reperfusion injury (54). These conditions are also associated with the production of reactive oxygen species, which can chemically quench NO and/or damage the endothelium and thus impair NO production.

Anthocyanin concentrations in the bloodstream are too low to directly contribute to in vivo quenching of reactive oxygen species even though they exhibit superior antioxidant potential to classic antioxidants such as butylated hydroxyanisole. They may, however, be adequate to improve endothelial function by influencing NO levels. Chokeberry and bilberry anthocyanin-rich extracts have the ability to prevent loss of endothelium-dependent, NO-mediated relaxation in porcine arteries in vitro at a level that roughly reflects that seen in several studies to exist in the human plasma after consumption of these compounds (55).

In a study by Youdim et al. (56), 4 anthocyanins isolated from elderberries were incorporated into the plasma, lemma, and cytosol of endothelial cells in vitro to directly examine their role. The results from this study indicate that anthocyanins can be directly incorporated into endothelial cells and produce significant oxidative stress protection (56). Delphinidin provided endothelium-dependent vaso-relaxation in the rat aorta comparable to that of red wine polyphenols (57). A similar finding with black currant concentrate was reported in rat aorta rings in vitro (58). Upregulation of eNOS in bovine endothelial cells after a 6-h exposure to 0.1 μmol/L cy-3-glu has been reported. In addition, 12-min exposure of bovine endothelial cells to cy-3-glu phosphorylates NOS and enhances NOS activity (59). Pelargonidin inhibits inducible NOS and mRNA expression as well as the production of NO in a dose-dependent manner in macrophages exposed to the inflammatory stimulus LPS (60). Other fruit pigment preparations have been shown to produce endothelium-dependent relaxation of the arteries; however, these effects have largely been confined to the pigments of red wine and grapes (57,61–64). Protection from heart

attacks through administration of grape juice and red wine has been strongly tied to the ability of anthocyanins to reduce inflammation, inhibit platelet formation, and enhance NO release (65).

Mazza et al. (17), using the oxygen radical absorbance capacity assay, found that the concentration of anthocyanins in the serum was directly correlated to the serum antioxidant capacity when adult males were supplemented with 1.2 g of anthocyanins from freeze-dried blueberries. This change in antioxidant capacity suggests that anthocyanins and their decretory products may play an important role in decreasing the production of superoxide by NADPH oxidase in addition to other possible mechanisms. A decrease in NADPH oxidase activity can lead to an increase in serum antioxidant capacity. It has been proposed that eNOS metabolism, rather than general antioxidant activity, is a major target of flavonols, a similar class of flavonoids, and that NADPH oxidase activity is a crucial site of action (66). The same theory could hold true for anthocyanins because of their similarity in chemistry.

Cytokines and chemokines. Cytokines are mediators of local and intercellular communications required for an integrated response to a variety of stimuli in immune and inflammatory processes. Different cytokines are associated with inflammatory disease, with a clinical outcome partially determined by the balance between proinflammatory and antiinflammatory cytokines (67). The analysis of structure-activity relationships among flavonoids suggests that 4 hydroxylations at positions 5, 7, 3^1, and 4^1, together with a bond at the $C_2 - C_3$ and the B-ring attachment at the C_2 position, seem necessary for the highest proinflammatory cytokine expression (68). Chemokines are small cytokines that play a significant role in controlling leukocyte migration. Monocyte chemotactic protein 1 (MCP-1) is a chemokine secreted by activated macrophages and endothelial cells whose production is upregulated in both acute and chronic inflammatory diseases. MCP-1 is known to mediate the signaling of macrophages to sites of inflammation in the body and is directly involved in the development of atherogenesis. Anthocyanins may protect against TNFα–induced MCP-1 secretion in human endothelial cells (69). Rats administered 4% freeze-dried whole blueberries in a high-fat diet showed a significant decrease in the proinflammatory TNFα, MCP-1, and IL-10 molecules. These results were not demonstrated in rats fed a low-fat diet (24). The level of MCP-1 released by adipocytes is significantly greater in obese mice than in nonobese mice and when adipocytes are co-cultured with macrophages (70). Treatment of endothelial cells with cy-3-glu and pel-3-glu has been reported to inhibit the production of cytokines and matrix metalloproteinases (MMP), including MMP-1 and MMP-9 (71).

NF-κB and other signal transduction pathways. In several studies, the suppression of proinflammatory chemokines, growth factors, and adhesion molecules was associated with an inhibition of NF-κB activation (72–74). NF-κB, an oxidative stress-sensitive transcription factor that controls expression of genes involved in the inflammatory response, is the most widely studied inflammatory mediator. Several NF-κB–related proinflammatory chemokines, cytokines, and mediators of inflammatory responses were shown to decrease in the plasma of healthy adult participants after supplementation with anthocyanins in parallel-designed, placebo-controlled, clinical trials, suggesting mediated inhibition of NF-κB activation by anthocyanins in vivo (51,75,76). Direct inhibition of LPS-induced NF-κB transactivation by anthocyanins was observed in human monocytes (51). Similarly, red wine has been reported to inhibit NF-κB production of proinflammatory factors in endothelial cells and inflammatory cells (77,78). In humans, treatment with lyophilized grape powder for 4 wk was associated with a reduction in NF-κB (79).

Treatment of human umbilical vein endothelial cells with anthocyanins regulated cholesterol distribution by interfering with the recruitment of TNF receptor-associated factors-2 in lipid rafts, thereby inhibiting CD40-induced proinflammatory signaling (71). The anthocyanin delphinidin has been shown to decrease the extent of apoptotic and necrotic cell death in cultured cardiomyocytes and to reduce infarct size after ischemia in rats. This process is mediated by the inhibition of signal transducers and activators of transcription 1 activation (80).

Adhesion molecules. Vascular endothelial cells line the luminal side of blood vessels and mediate interactions among the blood vessels, blood, and tissue (81). Endothelial cells recruit leukocytes by selectively expressing adhesion molecules on their surface as a response to proinflammatory stimuli such as TNFα and LPS. Flavonoids, including anthocyanins, seem to modulate this type of monocyte adhesion during the inflammatory process by decreasing their expression by endothelial cells.

Anthocyanins suppress the induced secretion of several molecules related to inflammatory modulation, specifically vascular endothelial growth factor and intracellular adhesion molecule-1 in cellular models (56,73,82). In a study of 9 major red wine polyphenols, only the anthocyanins delphinidin and cyanidin inhibited the platelet-derived growth factor AB-induced expression of vascular endothelial growth factor by preventing activation of redox-sensitive p38 MAPK and c-Jun N-terminal kinase pathways (83). This study suggested a crucial role of the hydroxyl residue at position 3 of the B-ring, because significant inhibitory effects were not shared by other anthocyanin compounds such as malvadin and peonidin, which contain a methoxyl residue at position 3 (83).

Anthocyanins may also protect against adhesion molecule production induced by activated platelets. An investigation of optimal platelet function showed that anthocyanins and their colonic metabolites inhibit thrombin receptor-activating peptide–induced platelet aggregation but did not influence platelet reactivity when strong agonists such as collagen and ADP were present (84). Antithrombotic properties were exhibited by 10 μmol/L dihydroferulic acid and 3-(3-hydroxyphenyl) propionic acid (colonic metabolites) as well as 1 μmol/L del-3-rut and a mixture of all compounds (84).

CRP. Low-grade chronic inflammation signaled by increased levels of CRP has been recognized as an independent risk factor for CVD (25). CRP is an acute phase reactant whose elevation in the serum is considered an indicator of chronic inflammation and whose interaction with endothelial cells may be one mechanistic link to atherosclerosis because it induces adhesion molecule expression (85). Analyses of NHANES data show a significant inverse association between serum CRP and anthocyanin intakes among adults in the United States (86). Data from the USDA flavonoid databases matched with a 24-h dietary recall indicate that anthocyanidin intakes were inversely associated with serum CRP concentration (86). A recent clinical study using anthocyanin-rich sweet cherries showed a decrease in serum CRP after 4 wk of intervention (76).

Conclusion

Knowledge of anthocyanin metabolism, absorption, and bioavailability as related to CVD has increased tremendously over the last decade; however, much work remains to achieve definitive conclusions about the potential of anthocyanins in CVD protection. The need for future research in this area is clearly evident. Although

experimental studies seem to demonstrate the potential of anthocyanins to influence many CVD-related biomarkers, epidemiological evidence remains promising but insufficient. A large prospective study of the cardio-protective effects of anthocyanins should be conducted with comprehensive information about their dietary intake. Anthocyanins seem to have a clear effect on endothelial function and proinflammatory markers, even if most of the effects are reported using in vitro assays. The relevance of many in vitro studies to the in vivo situation needs to be confirmed, because many in vitro studies apply high concentrations of anthocyanins that far exceed the level observed in vivo; however, some studies do achieve results at comparable levels.

Isotopic labeling of anthocyanins would generate better knowledge about the way these phytonutrients are metabolized and absorbed in the gut and/or in which tissues they accumulate throughout the body. Increased studies involving metabolism of anthocyanins by the gut microbiota are needed to better understand the bioactivity and bioavailability of these compounds. Intervention studies of participants at risk for CVD or related pathologies compared with healthy human participants are needed to properly determine the effect of anthocyanins on CVD-related biomarkers. Large-scale, long-term, randomized, placebo-controlled human trials are needed to validate the amount of anthocyanins required to achieve "optimal" vascular health.

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