Biological Properties of Anthocyanin Pigments in Blood Oranges

Subjects: Biochemical Research Methods

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Anthocyanins are natural pigments that give a red, purple, and blue color to many plant, flower, fruit, and vegetable species. Their presence within the genus *Citrus* was first reported in 1916, and it is well-known that the red color of the flesh and rind of blood (red or pigmented) oranges (*Citrus sinensis* L. Osbeck) is due to the presence of anthocyanins. They are also present in the young shoots, flowers, and peel of lemon (*Citrus limon* (L.) Burm. f.), citron (*Citrus medica* L.), and other citrus species.

Keywords: anthocyanins ; citrus ; blood orange ; antioxidant activity

1. Introduction

Anthocyanins are an extensive group of plant pigments belonging to the flavonoid family. They are responsible for the red, purple, and blue colors in some fruits and vegetables. Within the *Citrus* genus, anthocyanins are found mainly in the flesh and sometimes the rind of orange varieties (*Citrus sinensis* L. Osbeck) called blood or pigmented or red oranges [1]. Moreover, the anthocyanins are even expressed in the young shoots and floral tissues of lemon (*Citrus limon* (L.) Burm. f.), citron (*Citrus medica* L.), and other *Citrus* species [2]. The presence of pigmented stigmas containing anthocyanins in young flowers from the 'Moro' orange tree has been recently discovered at a commercial orchard located in Sicily (Italy) [2]. Since it is known that floral tissues from sweet oranges do not accumulate anthocyanin pigments, this is the first time that this phenomenon has ever been reported.

The geographical origin of the *Citrus* species was in southeast Asia, and they were later introduced into Europe [3]. Specific regions include the Yunnan province of southwest China, northeastern India in the Himalayan foothills, and Myanmar [4]. Hodgson [1] has suggested that blood oranges originated from Asia, although it has long been thought that these pigmented varieties derived from a genetic mutation that occurred in the Mediterranean area. Molecular analyses conducted by Butelli et al. [5] demonstrated that most of the current blood orange cultivars grown in China are of direct or indirect Sicilian origin; and an old Chinese blood orange variety named Jingxian [6], which is not widespread because of its poor fruit quality and the presence of many seeds in the fruit, is today the only blood orange of Chinese origin. Therefore, even if blood oranges originated in Asia, the long process of *Citrus* domestication and recent propagation techniques adopted, such as grafting on selected rootstocks or selection of superior branches derived from bud mutations, have led to the spread of a specific phenotype; thus, today blood oranges grown in different areas of the planet all have Mediterranean origins.

The most cultivated of the blood orange cultivars in Italy is the 'Tarocco' variety, followed by 'Moro' and 'Sanguinello' [7][8]. There are also varieties of blood oranges that are not very widespread, such as 'Sanguigno', 'Doppio Sanguigno', etc., from which it is thought that the 'Tarocco', 'Moro', and 'Sanguinello' varieties derive [9].

Since 1973 the CREA-Research Center for Olive, Fruit and Citrus Crops (CREA-OFA) in Acireale (Italy) has been working on a genetic improvement program for existent blood (red) orange cultivars, also targeting the production of new pigmented citrus hybrids with increased size, ease of peeling, and new original sensorial characteristics [10]. New red-fleshed mandarin-like hybrids have been produced by crossing mandarin (*C. deliciosa* Ten.) or some clones of clementine (*C. clementina* ex Tan.), used as female parents, with different cultivars of blood oranges employed as male parents. Phytochemical studies of these new pigmented citrus hybrids have showed that some traits, such as the anthocyanin profile, were different with respect to their male parent, and in some cases an increase in the expression of some characteristics of the progenies was observed [11][12].

Many studies in recent years have shown that anthocyanins accumulated in leaves, fruits, and other plant tissues play an important role in improving the photoprotective functions and act as reactive oxygen species (ROS) scavengers, light screens, and metal chelators [13][14]. Moreover, studies have demonstrated that certain anthocyanins have antiviral, antibacterial, and fungicidal activities [15][16][17]. The contribution of anthocyanins in plant resistance to biotic stresses is poorly understood compared with that in plant resistance to abiotic stresses. Lin et al. [18] have characterized the function of anthocyanins in protecting fruit from green mold, the major postharvest disease of citrus fruit. Compared with other oranges, 'Tarocco' orange, one of the most important blood oranges enriched in anthocyanins, showed reduced susceptibility to the necrotrophic fungus Penicillium digitatum (Pd), which causes citrus postharvest green mold. The fungal infection induces anthocyanin production by activating the expression of several genes in the biosynthetic pathway [19]. As health-promoting plant secondary metabolites, anthocyanins are involved in protection against numerous human diseases that are associated with their antioxidant properties [20]. Blood orange juice has demonstrated an important antioxidant activity owing to the presence of anthocyanin and other phenols [21]. Available research data have revealed that the consumption of blood orange juice may produce positive effects in preventing chronic pathological conditions such as cardiovascular diseases and many types of cancers [22].

2. Biological Activity

Citrus fruits are rich in flavonoids, a wide group of phenolic compounds whose biological activity has been extensively recognized in literature. Numerous research studies have pointed to their antiviral, antimicrobial, anti-inflammatory, anti-ulcerative, and anti-allergenic properties [23][24]. Flavonoids can exert their antioxidant activity in many ways, including radical scavenging such as anti-lipoperoxides and metal chelating agents. Four types of flavonoids (flavanones, flavones, flavanols, and anthocyanins) are present in the genus *Citrus*, the latter being present exclusively in blood oranges and in the young shoots and flower tissues of lemon (*C. limon* (L.) Burm. f.), citron (*C. medica* L.), and other *Citrus* species [2]. These phenolic compounds protect plants exposed to biotic or abiotic stresses such as infections, injuries, UV radiation, pollutants, and other adverse environmental conditions owing to their wide antioxidant properties. These bioactives not only play a significant physiological and ecological role, but they are also commercially relevant because of their wide range of applications in the food, cosmetics, and pharmaceuticals industries. Notably, much *Citrus* flavonoid activity appears to have an impact on blood and micro-vascular endothelial cells, and not surprisingly, the major fields of research on the biological activity of *Citrus* flavonoids are inflammatory and cancerous diseases [24][25].

Anthocyanins are considered to be the most significant subclass of flavonoids because of their high antioxidant activity and other physico-chemical and biological properties [22][24]. This unique group of phytochemicals, consumed in fresh fruits or their derivatives, has been recognized as a highly functional ingredient and for its positive health effects (**Table 1**).

Table 1. *Citrus* anthocyanins and their positive health effect.

Author(s),. Year	Risk Factor	Effect of *Citrus* Anthocyanins	Disease
Grosso et al., 2013 [22]; Buscemi et al., 2012 [26]; Silveira et al., 2015 [27]; Cassidy et al., 2011 [28].	Blood pressure	Decreased vascular inflammation	Heart disease (atherosclerosis, high systolic blood pressure, high level of chlolesterol, hypertension, ischemic heart)
Grosso et al., 2013 [22]; Silveira et al., 2015 [27].	Cholesterol	Help cholesterol level by raising HDL and lowering LDL cholesterol	Heart disease (high level of LDL cholesterol, stroke)

Author(s),. Year	Risk Factor	Effect of *Citrus* Anthocyanins	Disease
Grosso et al., 2013 [22]; Bonina et al., 2002, 2005, 2008 [29][30][31].	Oxidation	Decrease oxidation	Heart disease (atherosclerosis, lipid oxidation, oxidative stress)
Grosso et al., 2013 [22]; Cerletti et al., 2015 [32].	Inflammation	Decrease inflammation	Heart disease (atherosclerosis, oxidative stress, vascular stiffness), obesity (high level of abdominal fat)
Talagavadi et al., 2016 [33]; Titta et al., 2010 [34]; Fabroni et al., 2016 [35]; Salamone et al., 2012 [36].	Abdominal fat	Enhanced lipase enzyme activity	Obesity (high blood lipid levels), fatty liver (hepatic steatosis), type 2 diabetes (uncontrolled oxidation of lipids), metabolic syndrome (abdominal obesity, high blood sugar, high cholesterol, hypertension)
Grosso et al., 2013 [22]; Silveira et al., 2015 [27].	Blood levels of glucose	Improved insulin sensitivity	Type 2 diabetes (oxidative damage, high glucose level, high blood pressure)
Bonina et al., 1998 [37]; Puglia et al., 2014 [38]; Cardile et al., 2010 [39].	UV radiations	Photoprotective, anti-ageing	Oxidative damage (skin rash, photo-oxidative skin lesions, allergic contact dermatitis, psoriasis, atopic dermatitis)

These bioactive compounds can enhance human health in many ways, and a major one is through their ‘antioxidant’ effects. However, as numerous studies in this field have shown, the health benefits attributed to the compounds present in blood oranges are not only attributable to their antioxidant activity.

New research has demonstrated that these compounds also have anti-inflammatories, anticarcinogens, and many metabolic effects that help protect against diabetes, obesity, risk factors for cardiac disease, and cancerous cell development [22][27][29][34][35][36].

2.1. Metabolic Syndrome, Weight Management, and Obesity

The metabolic syndrome is a state characterized by abdominal obesity, high blood sugar and cholesterol, and hypertension, all of which are major risk factors for type 2 diabetes and cardiac disease. The anthocyanin pigments present in blood orange fruits improved insulin resistance, lowered cholesterol and systolic blood pressure, decreasing the risk factors for metabolic syndrome [27]. A 2010 study [34] indicated that blood orange anthocyanins can impair fat cell function and are therefore less likely to be stored as fat. A group of mice were given a regular feeding with the addition of water, blood orange or Washington Navel (blond) orange juice. Another group received a fat-rich diet accompanied by one of the same three beverage alternatives. The group of mice drinking blood orange juice in addition to the standard diet was found to gain less weight with no impact on both blood glucose and lipid levels than those drinking blond orange juice or even water. This was despite the increase in caloric intake from the sugar content of the juice. Moreover, blood orange juice significantly reduced or eliminated weight gain in mice on a fat-rich diet, with a 50% registered decrease in body fat [34]. The intake of anthocyanin-rich blood orange juice also increased the insulin susceptibility in mice through activation of the AMP-activated protein kinase, an established and recognized therapy for diabetes [33]. Treating diabetic patients with an anthocyanin-rich red (blood) orange extract can be therapeutically beneficial to protect them from the complications of diabetes that are caused in part by uncontrolled oxidation of lipids [29]. Pancreatic lipase inhibition, which divides triglycerides into glycerol and fatty acids, is at present a major treatment for obesity. In order to identify other sources for preventing and treating obesity, lipase inhibition using extracts containing anthocyanin was investigated [35]. With regard to inhibition efficacy, the extract enriched with cyanidin 3-glucoside (derived from 'Moro' blood oranges) showed the highest in vitro inhibitory efficiency on pancreatic lipase. This result confirmed that anthocyanins are a more effective lipase inhibitor than other natural polyphenols [35].

A follow-up study [36] aimed to establish whether 'Moro' orange juice can enhance hepatic lesions in mice affected by dietary-induced obesity. The results demonstrated that 'Moro' orange juice neutralizes hepatic steatosis in mice suffering from food-induced obesity, representing a dietary option for the prevention of fatty liver.

2.2. Heart Health

The usual consumption of foods high in anthocyanins, including blood (red) oranges, also reduces the risk of heart disease [22][27]. A 2012 study [26] examined the effect of red orange juice consumption on the oxidative stress and inflammatory markers in patients with high cardiovascular risk. The blood flow of the treatment group that received red orange juice was considerably enhanced, and a number of inflammatory biomarkers, including the C-reactive protein, notably declined. These findings suggested an anti-inflammatory effect of red orange anthocyanins that benefits the patient's cardiovascular system. Dietary intake of anthocyanins may also help protect against hypertension (a relevant risk factor for heart disease) [28]. This study suggested a daily intake of red orange anthocyanins between 12.5 to 15 mg to have a positive impact on reducing and preventing high blood pressure. Furthermore, in healthy patients, the concurrent intake of anthocyanins from red orange juice may prevent the low-grade inflammatory response caused by a fatty meal at cellular and possibly vascular function levels [32], and the supplementation of anthocyanins through blood orange extract is able to reduce oxidative stress, providing protection against its undesirable consequences on human health [30][31].

2.3. Anti-Ageing and Photoprotective Effects

Skin is the primary line of defense in the human body, which means it is constantly exposed to a wide range of chemical and physical attacks such as atmospheric pollution and UV rays. A study [38] assessed the protective and anti-ageing effects on the skin of a dosage equal to 100 mg/day of a standardized blood orange extract. This dose amounted to about 3 mg of daily anthocyanins intake. The findings showed a notable decrease in the level of skin rash (redness), with a mean reduction of 40%, as well as pigmentation of the cutaneous spots was observed to decrease from 27 to 7% in subjects exposed to the UV radiation from a solar lamp during the period of supplementation with red orange extract. These experiments showed that blood (red) orange extract was able to compensate for the adverse effects of UV rays as natural sun protection. An analogous study using the same standardized red orange extract (ROE) rich in anthocyanins [37] demonstrated the high antioxidant capacity of ROE in vitro, with a direct relationship between ROE scavenger efficiency and its level of antioxidants. In in vivo experiments, ROE provided effective protection against photo-oxidative skin lesions when topically used immediately after dermal exposure to UVB rays. Moreover, the anti-inflammatory effects of ROE were evaluated on the human keratinocytes that contribute to the physical health of the skin [39]. The results indicated that ROE exhibits anti-inflammatory properties, reducing the adverse consequences of certain skin pathologies such as allergic contact dermatitis, psoriasis, and atopic dermatitis.

2.4. Anticancer Activity

Oxidative stress is an event caused by a disproportion between production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of a biological system to remove these reactive products [40].

It is responsible for a chronic inflammatory state that plays an important role in neurodegenerative diseases and the development of cancers. Carcinogenesis is a multistep process activated by genetic alterations that modify different signal transduction pathways and cause the gradual transformation of a normal cell into a tumor cell. The signal transduction pathways involved in the formation of tumors often interact with each other, expanding the oncogenic signals necessary for the progression of the malignant form [41]. Available scientific studies have proved the advantageous effects of the presence of anthocyanins in fruits and vegetables in the prevention of tumor diseases (**Table 2**). Tsoyi et al. [42] investigated the protective effect of anthocyanins on UVB-induced apoptosis. UVB irradiation-induced apoptotic cell death was inhibited by topical application of anthocyanins in hairless mice. This study suggested that anthocyanins may be useful natural products to modulate UVB-induced photoagin

Table 2. *Citrus* anthocyanins and their anticancer activity.

Author(s), Year	Risk Factor	Effect of *Citrus* Anthocyanins	Disease
Tsoyi et al., 2008 [42]	UVB radiations	Photoprotective,	Photocarcinogenesis, apoptotic cell death
Jang et al., 2008 [43]	Intracellular oxidative damage	Anti-carcinogenic	Colon Carcinoma, angiogenesis
Li et al., 2010 [44]	Inflammation	Reduced risk of prostate or pancreatic cancer	Prostatic or pancreatic cancer
Forester et al., 2014 [45]	Inflammation	Decreasing cell viability, cell cycle arrest and apoptosis	Colon cancer
Jang et al., 2010 [46]	Inflammation	Reduce prostatic hyperplasia	Prostatic cancer
Grosso et al., 2013 [22]	Cell mutation	Anti-carcinogenic, anti mutagenic	Colonic adenocarcinoma, melanoma, vulva carcinoma

Anthocyanins have been extensively studied for their anticancer characteristics as well as anti-angiogenesis, based on in vitro and cell culture studies and animal models [47]. Endothelial cells are the principal cells involved in the angiogenesis process. Angiogenesis is the key to cancer progress, and it is an important step in the transition of tumors from a benign state to a cancerous one. In studies on the human colon tumor HT-29 cell line, the authors of [43] proposed phosphoglycerate kinase 1 (PGK1) as a possible biomarker of intracellular oxidative damage. Cells exposed to 50 µM H_2O_2 for 24 h showed significant expression of PGK1. Additionally, cells treated with delphinidin had attenuated expression of protein. High levels of PGK1 are associated with cancer survival and angiogenesis. These studies proposed that the antioxidant potential of delphinidin could contribute to an anti-cancer approach.

Citrus fruits such as oranges, lemons, tangerines, grapefruits, and limes are widely consumed worldwide. They are rich in bioactive compounds such as carotenoid, folate, vitamin C, limonoids, and flavonoids, which have been demonstrated to have anticancer effects. Various reviews of citrus fruit consumption showed an inverse correlation with the risk of esophageal, gastric, breast, bladder, oral, and pancreatic cancers [44][48][49][50].

Different biological activities of anthocyanins have been studied with the aim of preventing cancer. Grosso et al. [22] discussed the main health-related characteristics of blood (red) oranges that include anticancer, anti-inflammatory, and cardiovascular protection properties, and the effects on health of the main constituents of blood oranges. They specified the mechanism of action of the main components of blood oranges and reported an antimutagenic activity of anthocyanins. Additionally, a cyanidin–DNA copigmentation complex was identified as inhibiting the reverse mutation induced by heterocyclic amines in microsomal activation systems. The antimutagenic action was demonstrated in a study on colorectal carcinogenesis inducted by 1,2-dimethylhydrazine (DMH), confirming a previous study in which juice or extracts of plants or fruits containing high amounts of anthocyanins acted as inhibitors of heterocyclic amine mutagenesis [51]. Forester et al. [45] also described the positive effect of anthocyanin metabolites decreasing cell viability and causing cell cycle arrest and apoptosis in colon tumors. In oral and cervical cancer, the invasion of SCC-4 cells was diminished after the treatment with peonidin 3-glucoside and cyanidin-3-glucoside [52]. Jang et al. [46] studied the effects of anthocyanin on a rat model of benign prostatic hyperplasia (BPH) finding that the injection of testosterone developed prostatic hyperplasia as observed histologically during the tests; it was demonstrated that after anthocyanin treatment the average prostate weight in the BPH-induced group was significantly higher than in the control group, whereas the prostate weights in the anthocyanin-administered groups were significantly lower than in the BPH-induced group. It was concluded that the anthocyanin administration helped prevent this alteration. In addition, apoptotic body counts were significantly higher in groups receiving anthocyanin than in the BPH-induced group. These results suggested that the anthocyanin supplementation may be effective in BPH, and this experiment could be the basis for the clinical application of these compounds. Moreover, some authors have confirmed that the activity of anthocyanins is not mainly due to the compounds themselves; rather, it is the synergetic effect of anthocyanins and other phenolic compounds proving essential for the prevention of diseases. In vitro studies concluded that bacterial metabolism involves the splitting of glycosidic linkages and breakdown of anthocyanidin heterocycle, forming phenolic acids such as protocatechuic, vanillic, syringic, caffeic, and ferulic acids, aldehydes, and their subsequent methyl, glucuronide, and sulfate conjugation [53]. It is conceivable that the observed benefits of consuming anthocyanin-rich foods are due to the complex mixture of metabolites that remain in tissues and biological fluids for a longer time and in higher doses than the parent anthocyanins.

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