Comprehensive REVIEWS in Food Science and Food Safety

Plant Betalains: Safety, Antioxidant Activity, Clinical Efficacy, and Bioavailability

Mohammad Imtiyaj Khan

Abstract: Betalains are accepted food additives derived from vacuoles of plants belonging to about 17 families in the order Caryophyllales. These pigments are composed of a nitrogenous core structure, betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid]. Betalamic acid condenses with imino compounds (cyclo-DOPA and/or its glucosyl derivatives) or amines and/or their derivatives to form violet betacyanins (for example, betanin) and yellow betaxanthins (for example, indicaxanthin), respectively. Till date, structures of 75 betalains have been elucidated from plants under the order Caryophyllales. The extracted betalains are safe to consume and they act as micronutrients in the body. *In vitro* studies to highlight radical-scavenging activity, cell culture studies to assess cytotoxicity and absorption of betalains, and proven clinical efficacies are compiled in this review. The literature on biological activity has not been analyzed for a synthesis of safety, clinical efficacy, and bioavailability to arrive at the concentrations required for the purported health benefits. Most betalains are under-utilized in pharmaceutical and cosmetic preparations due to poor stability and lack of scientific reports highlighting their superior tinctorial strength including flourescence, water solubility, and functional value alongside their bioavailability. This is the first comprehensive review on the dietary safety, biological activity and bioavailability of betalains. Based on this review, for future debate and input from health professionals, a human daily intake of betanin and indicaxanthin can be proposed at 100 and 50 mg, respectively.

Keywords: betanin, caryophyllales, gomphrenin, indicaxanthin, intracellular signalling

Introduction

Betalains are vacuolar N-heterocyclic pigments having a core structure (protonated 1,2,4,7,7-pentasubstituted 1,7-diazaheptamethin system) referred to as betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid] (Figure 1A). Biosynthesis of this chromophore involves 2 enzymes: (1) a bifunctional cytochrome oxidase CYP76AD1 (and/or tyrosinase) having monooxygenase (De Loache and others 2015) and diphenol oxidase (Hatlestad and others 2012) activities, and (2) L-DOPA (L-3,4-dihydroxy-phenylalanine) ring opening enzyme DOPA dioxygenase (DOD) (Christinet and others 2004). First, tyrosine is hydroxylated to L-DOPA by CYP76AD1 (and/or tyrosinase), and the next step entails formation of betalamic acid catalyzed by DOD and further spontaneous cyclization. L-DOPA also gives rise to *cyclo*-DOPA through oxidation catalyzed by CYP76AD1 (and/or tyrosinase) and subsequent spontaneous cyclization. Spontaneous Schiff base condensation of betalamic acid with *cyclo*-DOPA and/or its glucosyl derivatives, and amines and/or their derivatives leads to formation of violet betacyanins and yellow betaxanthins, respectively. These pigments are accumulated in plants of about 17 families in the order Caryophyllales (Khan and Giridhar 2015). Spontaneous Schiff base condensation of betalamic acid with *cyclo*-DOPA and/or its glucosyl derivatives, and amines and/or their derivatives leads to formation of violet betacyanins and yellow betaxanthins, respectively (Figure 1B and C). Over the past 60 y, numerous researchers have generated a massive literature on the chemistry, biochemistry, ecophysiological factors affecting accumulation, mutual exclusiveness of betalains and anthocyanins, antioxidant activity, stability, and biological activity of betalains (Gandía-Herrero and others 2014; Khan 2015; Khan and Giridhar 2015). As a result, there are now 75 well-characterized plant betalains and the biosynthetic pathway is continually being upgraded (Khan and Giridhar 2015). Two decades ago, there were several reports of beeturia cases after consumption of betalain-rich foods (Mitchell 2001). However, such concerns failed to provide any scientific basis as beeturia turned out to be not controlled by genetic traits or a dose-dependent phenomenon, but rather a determinant of an individual's metabolic ability to process betalains in a given food matrix (Eastwood and Nyhlin 1995; Mitchell 2001). Following this, a number of studies on the safety, antioxidant and biological activities, and bioavailaibility of betalains were published. A recent review compiled the available literature on antioxidant activity, *in vitro* bioactivity, and clinical efficacy in animal models (Gandía-Herrero and others 2014). However, there is no compilation of the studies on dietary safety of betalains and biological activity alongside bioavailability, although there is sizeable body of literature. Therefore, there is an urgent need for streamlining the clinical efficacy studies of betalains and an interpretation of their results in correlation with bioavailability.

MS 20151557 Submitted 17/9/2015, Accepted 25/11/2015. *Authors are with Dept. of Biotechnology, Gauhati Univ., Guwahati 781014, India. Direct inquiries to author Khan (E-mail:* imtiyaj@gauhati.ac.in*).*

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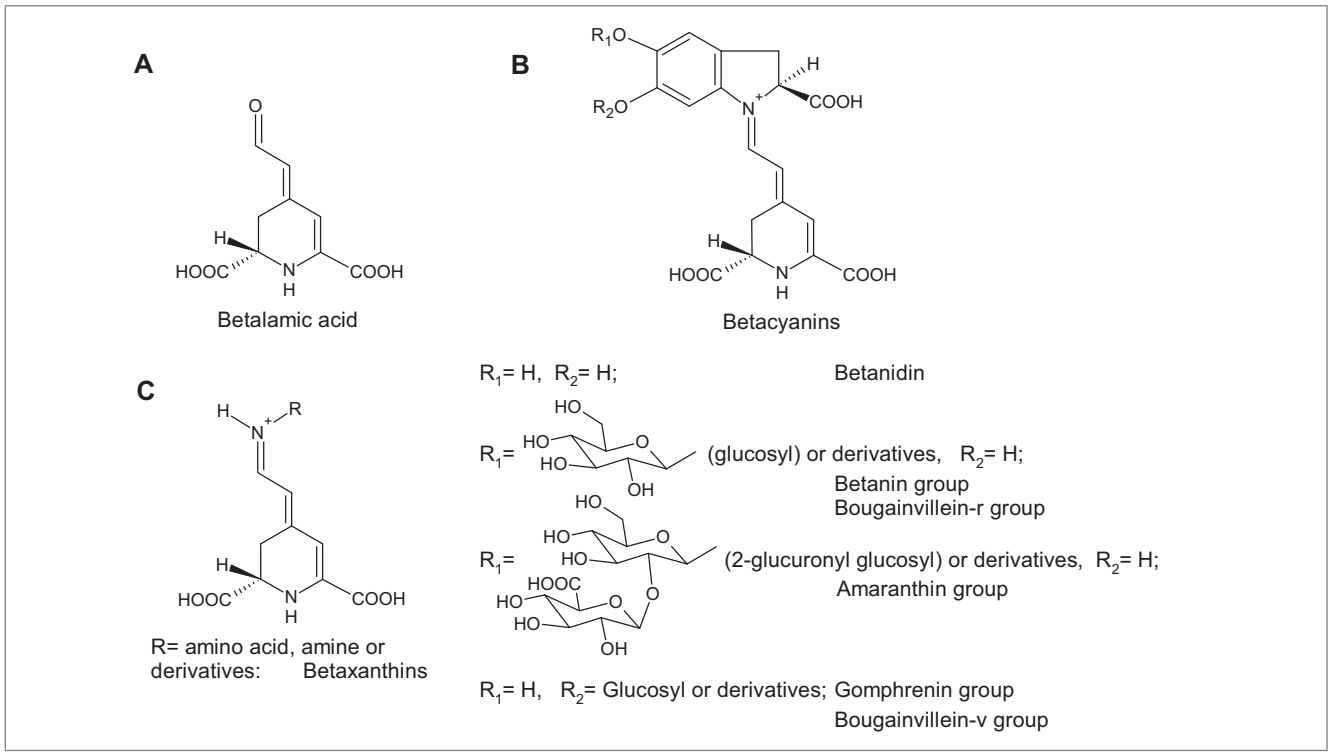


Figure 1–Representative structures of different classes of betalains. Adapted from Khan and Giridhar (2015). There are 32 betaxanthins, and 43 betacyanins including betalamic acid and betanidin. Among the betacyanins, 17 betanin type, 4 bougainvillein-r type, 5 amaranthin type, 4 gomphrenin type, 11 bougainvillein-v type structures are known till date.

In this regard, this review attempts to provide a critical commentary on the discrepancy concerning biological activity and bioavailability.

Dietary safety

Safety of betalains from dietary sources

Safety is a deciding factor for human consumption. In the earliest known report released by a FAO/WHO Expert Committee (FAO and WHO 1974), it was observed that there was inadequate information on safety and metabolism of beetroot pigments. Following this, studies on absorption, excretion, metabolism, and cardiovascular effects of beetroot extract were conducted (Krantz and others 1980). It was observed that orally administered betanin, the major pigment in red beet, was poorly absorbed, and the major portion of it was metabolized in the gut. In addition, betanin transiently increased blood pressure and heart rate. Further, beet pigments neither initiated nor promoted hepatocarcinogenesis (Schwartz and others 1983). Moreover, betanin inhibited IgE and IgG production suggesting lack of allergic response to the pigment (Pourrat and others 1987). Other aspects of safety such as absence of genotoxicity (Haveland-Smith 1981), mutagenicity, and short-term toxicity of beet betalains on *Salmonella typhimurium* and rats were documented (Elbe and Schwartz 1981). Recently, no mutagenicity in *S. typhimurium* was observed after exposure to betalain-rich extracts from fruits of some Cactaceae species (Zampini and others 2011). All these studies suggested the safety of betalains for human consumption, although studies on embryotoxicity, including teratogenicity and generational toxicity, are lacking. One concern related to the consumption of beet pigments was beeturia, the phenomenon of excretion of colored urine after ingestion of red beet. There had been many inconsistent reports on beeturia for some time. In a review by Mitchell (2001), it was systematically analyzed and explained that beeturia was neither a function of an individual's physiological constitution, nor yet proven to be under polymorphic genetic control. The available data at the time implied that beeturia was a function of quantity of consumption, coingestion with certain organic acids such as ascorbic acid and oxalic acid, and rate of gastric emptying. It was concluded that beeturia was just a food idiosyncrasy and not a physiological dysfunction (Mitchell 2001).

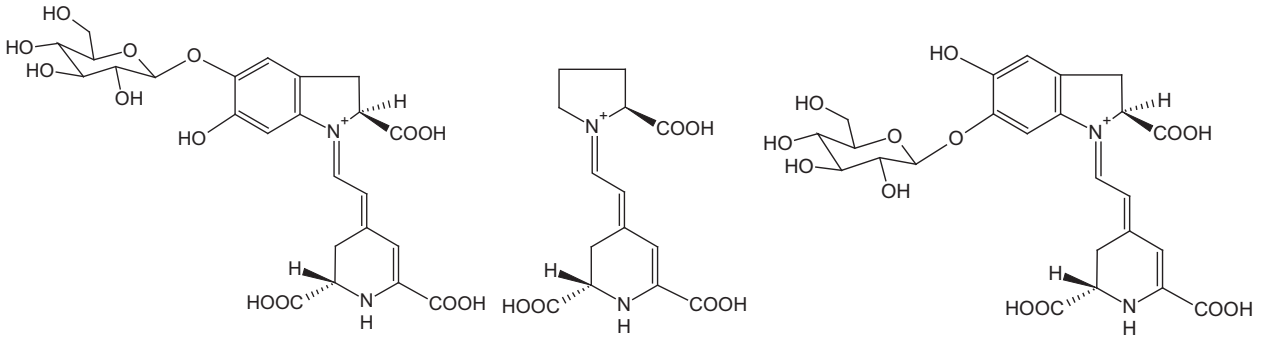
Despite increasing interest in the functionality of batalain sources, such as amaranth (Venskutonis and Kraujalis 2013) and quinoa (Graf and others 2015), most of the studies involving betalains to assess dietary safety, biological activity, and bioavailability (discussed in subsequent sections) have been conducted using red beetroot as source of the pigments because of its availability and bioaccessible functional components (Wootton-Beard and Ryan 2011; Vulić and others 2012, 2014; also see the references in subsequent sections). As a result, red beet betanin is a well-established red food colorant. Betanin (EEC No. E 162), approved as red food colorant by the European Union and under Section 73.40 in the Title 21 of the Code of Federal Regulations (CFR) by the Food and Drug Administration (FDA) in the United States, has been in use as colorant for dairy products such as yogurt, ice cream, ready-made frostings, and cake mixes (Delgado-Vargas and others 2000). Some other food products including candies, meat substitutes, powdered drink mixes, gravy mixes, marshmallow candies, soft drinks, and gelatin deserts are also colored with betalains (Delgado-Vargas and others 2000). It is assumed that less than 50 mg betanin/kg can produce the desired color, although some food scientists have suggested a higher quantity, namely, 0.1% to 1.0% (w/w) (Hendry and Houghton 1996). In recent times, many new sources of a wide range of betalains have been reported. Some of the sources are traditionally considered unsafe. For example,

one of the promising betalain sources, *Phytolacca decandra* berries have been known to contain toxic saponins, because of which the berries remain unutilized commercially (Forni and others 1983). In *Celosia argentea* var. *cristata*, the presence of a high level of dopamine (41.15 μmol/g fresh weight) (Schliemann and others 2001) may be a problem when consuming the betalain-rich extract of this plant. In view of the increasing number of structurally different betalains reported from plant sources, toxicological studies of betalain-rich extracts from *Myrtillocactus geometrizans* fruit (Reynoso and others 1999), *Rivina humilis* berry (Khan and others 2011), and *Hylocereus polyrhizus* fruit (Hor and others 2012) have been conducted on rodents and established that these extracts are not likely to produce any toxic effects in human beings. In addition, betalain extract from hairy roots of red beet did not cause any toxicity in rats (Khan 2006), thereby suggesting the possibility of sustainable production of safe betalains through biotechnology.

Safety of processed betalains

Apart from conventional extraction methods, betalain extraction has been successfully experimented with pulsed electric fields (PEF) (Zvitov and others 2003; Kannan 2011), which induced tissue damage or permeabilization resulting in release of pigments (Shynkaryk and others 2008). Similarly, microwaving (Cardoso-Ugarte and others 2014), microwaving coupled with enzyme treatment (Moussa-Ayoub and others 2011), aqueous 2-phase technique (Chethana and others 2007), γ-radiation (Nayak and others 2006), enzyme treatment (Krifa and others 1987), pressurized CO_2 (Nunes and others 2015), thermoultrasonication (Cruz-Cansino and others 2015), and some other novel methods (Thimmaraju and others 2003) have been used to extract betalains. Conventional methods of pigment concentration lead to formation of browning products at high temperature, whereas extended period of drying at ambient temperature may increase yellow colorant components (Shynkaryk and others 2008). To overcome these problems, novel techniques used for betalain concentration include fermentation (Castellar and others 2008; Klewicka and others 2012; Sravan Kumar and others 2015), osmotic distillation (Romero and others 2010), and convective drying (Gokhale and Lele 2012a). Fermentation for betalain concentration from *Opuntia stricta* fruit and the resultant pigment containing filtrate has been reported to be safe (Krifa and others 1987). Similarly, lactofermentation-concentrated betalains from *Beta vulgaris* root were safe (Klewicka and others 2012). Furthermore, betalains extracted after enzyme pre-treatment (Sembries and others 2006) and pulsed electric fields (Kannan 2011) have been observed to be safe. Except for these few reports, there is no report on safety, biological activity, and bioavailability of the betalains processed through novel methods. However, some of these processing technologies have been proven to be beneficial in enhancing antioxidant activity or biological activity of extracted betalains (Krifa and others 1987;Kim and others 2007; Kannan 2011; Lee and others 2012; Gokhale and Lele 2012b; Ravichandran and others 2013; Cruz-Cansino and others 2015; Nunes and others 2015; Sravan Kumar and others 2015) due to enhanced co-extraction of antioxidants such as flavonoids, and phenols. In addition, beetroot juice concentrates with high contents of neobetanin, a degradation product of betanin formed during processing, have been observed to lower postprandial insulin and glucose response (Wootton-Beard and others 2014). On the other hand, some of the common processing techniques used for preparing betalain concentrates such as spray-drying, freeze-drying, and air-drying have led to wide variations in pigment composition and other phytochemical profiles in the end-product (Nemzer and others 2011). In the wake of this, there is a need for scientific documentation of the safety, stability, and applicability of betalain-rich formulations produced through the various processing technologies, in particular, because of the conflicting claims of reduced stability (Nayak and others 2006), incomplete pigment extraction (Zvitov and others 2003; Nunes and others 2015), and low pigment content (Cruz-Cansino and others 2015) associated with some processing methods. Also, in the case of nonlactofermentation for processing of betalain−rich extracts (Sravan Kumar and others 2015), safety and flavor aspects of accumulation of fermentation end products and unwanted by−products need to be studied systematically.

Biological activity of betalains



***In vitro* radical−scavenging activity.** Betalain research has lagged behind compared to anthocyanins, which are considered as functional analogs of betalains, due to its restricted presence in the Caryophyllales order only. However, in the last 15 y, there has been renewed interest in betalains since their antiradical activity was characterized for the first time in 1998. Subsequently, many studies also have reported a correlation between radical−scavenging activity (RSA) and betalain content of crude plant extracts (Czapski and others 2009; Canadanovic-Brunet and others 2011; Vulić and others 2012; Vulić and others 2014) suggesting the possibility of using such extracts as bioactive food additives. Antiradical or antioxidant activity is a measure of the ability to delay or prevent oxidation of proteins, lipids, carbohydrates, and DNA caused by ROS and reactive nitrogen species (RNS) (Halliwell and others 1995). Antioxidants are considered as insurers of health owing to

their proposed radical–scavenging activity and related biological activities (Khan and Giridhar 2011). Depending upon the target substrate in a study, there are different model experimental systems to assess the degree of protection offered by antioxidants against oxidation induced by standard synthetic radicals such as $ABTS^{\cdot+}$, lipoperoxyl, and $DPPH^{\cdot}$. Owing to dissimilar experimental models followed by different researchers, there have been inconsistent results in terms of betalains' RSA. For example, Escribano and others (1998) used $ABTS^{\cdot+}$ to characterize RSA, whereas Zakharova and Petrova (1998) employed lipoperoxyl radical. The result was that the former study observed betacyanins as stronger radical scavengers than betaxanthins, whereas the latter study found the opposite. However, in both studies they observed pH dependence of the RSA, and it was ascribed to e^- donating capacity and subsequent stability of the radicals. Many parallel studies followed either lipoperoxyl (Kanner and others 2001; Tesoriere and others 2007) or $ABTS^{\cdot+}$ line of work to assess RSA. In studies involving $ABTS^{\cdot+}$, betanin was 1.5 to 2 times more efficient as a free radical scavenger than anthocyanins at neutral or basic pH (Gliszczyńska-Świgło and others 2006). Strong electronic interaction between betalamic acid and the *cyclo*-DOPA-5-*O*-glucoside moiety contributed to the activity. Furthermore, the chemical basis of betanin's strong RSA was studied through phenolic O–H homolytic bond dissociation energy and ionization potential. The results revealed increased hydrogen and electron donating capacity at pH > 4, particularly in basic pH wherein mono-, di-, and tri-deprotonated forms are present (Gliszczyńska-Świgło and others 2006). Contrary to this, betanidin, the aglucone form of betanin, was reported to have strong RSA at pH 2–4, apparently because of the presence of the cationic form at acidic pH and the catechol substructure (Gliszczyńska-Świgło and Szymusiak 2006). Further support came from results obtained from voltammetric oxidation of betanidin and subsequent analysis of the products by LC-HRMS showing low oxidation potential, which meant that the pigment was a strong reducing agent (Wybraniec and others 2011). It was also observed that, among betacyanins such as betanidin, betanin, and phyllocactin, betanidin was the most potent antioxidant against peroxyl radical and nitric oxide indicating that glucosylation and further acylation reduces RSA of betacyanins (Taira and others 2015). However, in all these studies, there was no focus on the RSA of the core structure, betalamic acid. The gap was sealed by a later study on the resonating core structure of all betalains, betalamic acid, which was shown to exhibit a pK_a of 6.8 which explained the pH dependence of RSA of all betalains. Furthermore, the study reported that each molecule of betalamic acid could reduce 2 Fe^{3+} molecules to Fe^{2+} (Gandía-Herrero and others 2012). In other words, it could donate 2 electrons to an oxidizing agent. In the case of betaxanthins, the presence of catechol substructure results in potent RSA at higher pH (Gandía-Herrero and others 2010). In addition to the intrinsic RSA conferred by the core structure, the presence of phenolic hydroxy groups was also linked with higher RSA.

Among the studies that employed $DPPH^{\cdot}$, Cai and others (2003) observed higher RSA of gomphrenin than certain betaxanthins and betanin. The RSA was found to correlate with the number of hydroxyl and/or imino groups and their position in the aglucone structure of betalains. The study showed that RSA of betaxanthins was higher than betacyanins. This was consistent with recent results indicating EC_{50} of betaxanthins (0.11 μg/mL) and betacyanins (0.29 μg/mL) (Khan and others 2012). The authors also demonstrated that fractions of betaxanthins and betacyanins had better RSA against $DPPH^{\cdot}$ than ascorbic acid (also reported earlier by Cai and others 2003) and gallic acid. Similarly, betacyanins fraction was shown to be a better radical scavenger compared to flavonoid fractions of red pitaya flesh and peel (Wu and others 2006). Electron spin resonance coupled with spin trapping technique revealed very high RSA ($EC_{50} \sim 3$ μM) of betanin against $DPPH^{\cdot}$ (Esatbeyoglu and others 2014a). Contrary to this, in $ABTS^{\cdot+}$ line of work, it was considered that betanin had higher RSA than indicaxanthin, a betaxanthin pigment, owing to the monophenol nature of betanin and its oxidation products that acted as reducing intermediates conferring to the molecule a higher H^+ or e^- donation potential (Butera and others 2002). This was evident from the redox potential determined for the 2 compounds by cyclic voltammetry. Apart from the pH dependence of RSA, it was illustrated that the presence of phenolic hydroxyl group boosted, whereas glucosylation inhibited, but acylation had no effect on the activity or inhibited it (Gandía-Herrero and others 2010; Taira and others 2015). Based on these observations on structure–function relationship, miraxanthin V and betanidin are expected to possess the best RSA among betaxanthins and betacyanins, respectively, and owing to the presence of an imino substructure to facilitate extended e^- resonance, betanidin should exhibit higher activity than miraxanthin V. However, critical assessment of this extrapolation reveals that

(1) RSA assays reported so far involved a wide range of betalain concentrations at different pH conditions (Wettasinghe and others 2002; Gandía-Herrero and others 2013). Moreover, RSA against $DPPH^{\cdot}$ and reducing power (at pH 7) of betaxanthins were higher than betacyanins (Cai and others 2003; Khan and others 2012). Hence, there is no acceptable RSA that represents the actual antioxidant capacity of betalains.
(2) Stability of betalains is enhanced by glucosylation and acylation (Herbach and others 2006), which means that neither betanidin nor miraxanthin V will be stable enough to exhibit their RSA in a product formulation. Further, the RSA of most of the betalains, except betanidin, were shown to increase with pH, but, on the flip side, stability decreased at 7 < pH < 3 (Herbach and others 2006). Hence, paramount consideration should be given to stability factors while interpreting RSA to present a complete picture.

Meanwhile, betanidin, and miraxanthin have been successfully encapsulated separately to enhance stability without affecting their RSA (Gandía-Herrero and others 2013). However, *in vivo* the RSA and stability may be altogether different, which is not yet well understood. This notwithstanding, in all the studies reviewed in this section, there was unequivocal evidence that betaxanthins and betacyanins exhibit better RSA compared to ascorbic acid, Trolox (vitamin E analog), and gallic acid. Going by this, betalains are among the best antioxidant phytochemicals exhibiting desirable biological activities (see next section) limited only by their poor bioavailability (see subsequent section).

In vitro and in vivo biological activity

One of the earliest studies on biological activity of betalains revealed that red beet betanin when injected i.v. caused transient increase of blood pressure and heart rate (Krantz and others 1980). These effects on the cardiovascular system were not observed when betalains were administered through oral intubation, probably because of degradation in the GI tract (Reynoso and others 1999). Following this, the focus shifted to antioxidant activity–associated physiological functions. Protection against lipid peroxidation has

remained one of the essential factors to qualify as an antioxidant *in vivo*. Preliminary evidence came from an *in vitro* study that showed betanin and betanidin to be efficient inhibitors of membrane lipid peroxidation and LDL oxidation: betanin's efficiency was more than betanidin (Kanner and others 2001). In another study, betanin could inhibit 71% lipid peroxidation in a liposome system (Reddy and others 2005). This was reflected in betanin and indicaxanthin's ability to resist induced oxidative injury to LDL, wherein indicaxanthin, by virtue of its better bioavailability, was more efficient (Tesoriere and others 2004). In another study, betalain-enriched erythrocytes were more resistant to cumene hydroperoxide-induced membrane lipid peroxidation and subsequent hemolysis (Tesoriere and others 2005). Apart from lipid peroxidation, betanin protected H_2O_2– and ONOO-induced DNA damage through RSA (Sakihama and others 2012; Esatbeyoglu and others 2014a). In addition to RSA, protection of LDL oxidation *in vivo* by betalains could be through transactivation of paraoxonase 1 (PON1), an antioxidant enzyme produced in the liver (Esatbeyoglu and others 2014a).

Lipoxygenase (LOX) and cycloxygenase (COX) are bifunctional enzymes that convert arachidonic acid to leukotrienes and prostaglandins, which are chemical mediators of inflammation. Kanner and others (2001) presented data on soybean LOX inhibition by betanidin and betanin with an IC_{50} 0.25 and 0.5 μmol/L, respectively, which was higher than that of catechin (IC_{50} 1.1 μmol/L). Betanin inhibited COX-1 and 2 as well at 180 μmol/L upto 33% and 97%, respectively (Reddy and others 2005). In an *in vitro* study, among many betalain pigments, phenethylamine–betaxanthin was found to be the most potent inhibitor of COX, whereas betanidin (IC_{50} 41.4 μmol/L) was an efficient inhibitor of LOX (Vidal and others 2014). Through docking studies, it was apparent that betalains interacted with Tyr-385 and Ser-530 residues close to the active site of COX, and with substrate-binding amino acids of LOX. However, the study did not compare the efficiency to currently used inhibitors of the enzymes and did not explain why the IC_{50} of LOX inhibition was inordinately different from a previous report (Kanner and others 2001). Meanwhile, many researchers have investigated the effect of betalains against cellular mediators of inflammations. In endothelial cell culture studies, indicaxanthin and betanin significantly repressed intercellular cell adhesion molecule-1 (ICAM-1), which was expressed in response to an increase in intracellular oxidants triggered by cytokine treatment (Gentile and others 2004). As ICAM-1 is an important stimulus for the inflammation process, this study further strengthened the anti-inflammatory role of betalains: betanin was more efficient than indicaxanthin. Widening the scope, another study reported on the anti-inflammatory role of gomphrenin in murine macrophage cell cultures based on the suppression of induced nitric oxide production and decreased levels of prostaglandin E_2 (PGE_2) and interleukin (IL)-2β (Lin and others 2010). This observation was further supported by proportional gene expression patterns of inducible nitric oxide synthase, COX-2, IL-1β, tumor necrotic factor, and IL-6. Similarly, anti-inflammation related parameters were observed when indicaxanthin was added to Caco-2 cell cultures (Tesoriere and others 2014), and administered to rats with λ-carrageenin-induced pleurisy (Allegra and others 2014b). The mechanism possibly involves a reaction cascade culminating in production of anti-inflammatory cyclopentenone 15-deoxy-PGJ_2. To prove this, it was demonstrated, in macrophage cultures that lipopolysaccharide-induced prooxidant activity of indicaxanthin led to oxidation of membrane lipids to peroxides, which in turn, modulated PG biosynthesis (Allegra and others 2014a).

Hepatoprotective effect was suggested through induction of phase II enzyme quinone reductase by betalain-rich red beet extract (Wettasinghe and others 2002), particularly betanin (Lee and others 2005). Feeding trials involving rats showed that betalain-rich red beet juice mitigated hepatic toxicity caused by *N*-nitrosodiethylamine (NDEA), carbon tetrachloride (Kujawska and others 2009), and 7,12-dimethylbenz(a)anthracene (DMBA) as a result of improved antioxidant status and enhanced expression of phase II enzyme quinone reductase (Szaefer and others 2014). Liver toxicity was efficiently offset by the protective effects of betanin, such as reinstating cytochrome P450 enzyme expression elevated by toxicant treatment, improvement in liver redox status, and restoration of mitochondrial functions (Han and others 2014a,b). Concurring with this, even in *in vitro* studies, betanin's ability to modulate ROS production and DNA damage (Zielińska-Przyjemska and others 2012), and also inhition of nitrosative stress (Sakihama and others 2012), have been subsequently demonstrated. In the case of nitrosative stress, betanin's ability to inhibit (IC_{50} 19.2 μmol/L) $ONOO^-$-dependent nitration of tyrosine was convincingly higher than that of ascorbate (IC_{50} 79.6 μmol/L) (Sakihama and others 2012). Potent antioxidants are also required to counteract many other disorders characterized by elevated oxidative species which are dangerous to our physiological homoestasis. For example, exposure to ionizing radiation produces free radicals such as ROS and RNS that cause mutation or lethality, which is believed to be thwarted by antioxidants. Radioprotective efficiency of betalains was tested using red beet extracts, which could ameliorate the white blood cell count, micronuclei in polychromatophilic erythrocytes of bone marrow, antioxidative capacity, and spleen and thymus index in mice irradiated by ^{60}Co (Lu and others 2009). Among other examples of toxicity, substances such as D-galactose cause neurotoxicity characterized by oxidative stress in the brains of senescent mice. In these study models, administration of betanin improved endogenous enzymic and nonenzymic antioxidant profiles, thereby counteracting lipid peroxidation (Wang and Yang 2010). These findings underlined betacyanin's neuroprotective efficiency comparable to vitamin C that could delay signs of aging brain tissue to certain extent.

Comparable to that of the standard drug hydrochlorothiazide, diuretic effect of betalain-rich dehydrated *Opuntia ficus-indica* fruit extract, having a high reducing sugar content, was observed (Bisson and others 2010). A similar patented betalain formulation from red beet extract, having significantly high betalain content, (24.6%) was able to relieve 33% of osteoarthritis pain feelings in human volunteers (Pietrzkowski 2010). The inventor suggested that the formulation could be effective against various conditions such as acne, contact dermatitis, sinusitis, and allergy. However, the design of the experiments was open-type clinical discovery, but not a clinical efficacy study, since only 1 male and 1 female subject were employed for the acne-related study. Similarly, based on the clinical discovery concept, several betalain-rich formulations have been patented with claims of a wide range of health benefits. A solid betalain formulation from red beet was reported to be effective in maintaining serum lipid profile, including increasing HDL/LDL cholesterol ratio, reducing oxidized LDL concentration, normalizing blood glucose, inhibition of oxidative stress responsive transcription factor nuclear factor (NF)-κB, and increased expression of NAD-dependant histone deacetylase (a mammalian homolog of silent information regulator, SIR2) (Pietrzkowski and Thresher 2010). The authors contemplated that these observed effects could counter metabolic dysfunctions, because the efficiency, even at very low concentration, was much higher than resveratrol,

an approved therapeutic agent (Baur and Sinclair 2006). Partially supporting the claim of reducing blood glucose levels, betanidin, a red beetroot betalain, was shown to exhibit hypoglycemic effect in mice (Lugo-Radillo and others 2012).

Diabetes, caused by chronic hyperglycemia, has been recognized as a silent killer with an increasing number of diagnosis year after year. Since it has been generally believed that diabetes is a consequence of the stressful modern lifestyle, the prognosis is very much dependent on its management. Recent studies have reported betalains' ability to counteract diabetic complications as effects of chronic hyperglycemia. A short report on the hypoglycemic effect of betanidin in mice was published by Lugo-Radillo and others (2012). They observed a 50% reduction in the blood sugar levels of mice fed an atherogenic diet (24 wk) when supplemented with betanidin (9.6 mg) for the last 40 d. This study set off a few other investigations on hypoglycemic effect of betalain-rich preparations or purified betalains. In one of the studies, high neobetanin containing red beet juice fed human volunteers showed significant reduction in postprandial glycemic response in the first 15 min of intake (Wootton-Beard and others 2014). Interestingly, corresponding to the hypoglycemic response, insulin response was also observed suggesting reduced insulin requirement. The reason for insulin sensitivity was not confirmed as there was no significant difference in insulin sensitivity among the volunteers of control and test groups. However, it was apparent that betalain-rich meals significantly reduced glucose absorption. Lugo-Radillo and others (2012) used a high-fat diet known to induce atherosclerotic plaques. Although the researchers observed good glycemic response, they did not explore the effect of betanidin feeding on atherosclerosis-related pathophysiology which causes cardiac arrest. On the other hand, chronic hyperglycemia induced-complications also cause cardiac failure as a consequence of cardiac fibrosis triggered by very high accumulation and formation of collagen and its irreversible cross-links in extracellular matrix (ECM) as a result of advanced glycation end products (AGEs)-induced expression of cytokines such as profibrotic factor-transforming growth factor (TGF) β1 and connective tissue growth factor (CTGF) that stimulate fibroblasts to synthesize ECM (Candido and others 2003; Aronson 2003). AGEs intereact with multiligand receptors for AGEs or RAGEs to set off a signal that upregulates cytokines (Ihm and others 2010). In a diabetic model induced by feeding a high-fructose diet to rats, Han and others (2015) observed that betanin significantly decreased the levels of collagen cross-links and AGEs indicated by amelioration of soluble collagen ratio and reduced levels of Maillard reaction products such as protein glycation reactive intermediate (methylglyoxal), and advanced glycation end product (Ne-(carboxymethyl) lysine) and downregulation of RAGEs. In addition, redox balance was restored as the levels of antioxidants and enzymes improved as a result of attenuation of oxidative stress responsive transcription factor NF-κB. These observations suggested that betanin could control the causes of hyperglycemia—related cardiac fibrosis.

One of the most sought—after attributes in a pharmacoactive natural product is anti-cancer activity. This demand is driven by the increasing incidences of the complex disease as a side-effect of today's lifestyle and food habits. Anti-tumor activity of betanin against skin tumor initiated by DMBA and promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in rats was evident from the reduction of papilloma percentages after feeding betanin at 2.5 mg/100 mL water for 25 wk (Kapadia and others 1996). The researchers also observed 40% reduction in incidences of 4-nitroquinoline-l-oxide (4-NQO) initiated and 8% glycerol promoted pulmonary tumors. Besides, skin tumor initiated by DMBA and promoted by UV light-B and the associated symptom of splenomegaly were significantly inhibited by oral feeding of betanin (2.5 mg/100 mL water) (Kapadia and others 2003). In addition, this betanin dose could significantly inhibit other tumors simultaneously induced through different tumor initiator-promoter application. The authors also reported reduction in incidence and multiplicity of a 2-stage hepatocarcinogenesis model induced by the initiator NDEA and promoted by phenobarbital. Based on these findings, Kapadia and others (2003) suggested the possible use of betanin for combating malignancy. The molecular mechanism of anticancer activity appears to be that betanin reduces the number of CD31+ endothelial microvessels to inhibit angiogenesis, and increases the expression of caspase-3 to induce apoptosis (Zhang and others 2013). Earlier studies also had shown antiproliferative activity of betanin against various cell lines with IC_{50} values ranging from 142 to 164 μg/mL (Reddy and others 2005). Betanin's cytotoxicity (IC_{50} 40 μmol/L) against human chronic myeloid leukemia cell line (K562) was orchestrated through cellular events such as release of cytochrome C into the cytosol, poly (ADP-ribose) polymerase (PARP) cleavage, downregulation of Bcl-2, and reduction in mitochondrial membrane potentials (Sreekanth and others 2007). Consistent with these observations, betanin caused activation of initiator caspase-9, effector caspases-3 and 7, and cleavage of caspase-3 target PARP as evidenced in human lung cancer cell lines (Zhang and others 2013). Similarly, betanin/isobetanin treatment led to activation of both intrinsic and extrinsic apoptotic pathways in breast cancer cell line (MCF-7) (Nowacki and others 2015). All the above data, obtained from mouse studies, lung cancer cell lines, myeloid leukemic cell cultures, and breast cancer cell line clearly suggest anticancer effect of betanin through apoptosis. In other study models, such as stimulated neutrophils, a part of anti-inflammatory response, betanin significantly enhanced the activity of caspase-3 and its cleavage products, whereas in resting neutrophils, it had no effect (Zielińska-Przyjemska and others 2012). In contrast, indicaxanthin may be pro- or anti-apoptosis depending upon the concentration, pathophysiology, and affected cell type. For example, in macrophage cultures, indicaxanthin at 2.5 μmol/L was responsible for the chain of anti-apoptotic events, including inhibition of NADPH oxidase-4 (NOX-4) basal activity, as well as overexpression, repression of NF-κB, maintaining cellular redox balance and Ca^{2+} homeostasis, and maintaining mitochondrial membrane potentials (Tesoriere and others 2013a). In addition, dietary indicaxanthin (1 to 5 μmol/L) could significantly prevent death of erythrocytes or eryptosis related events and atherosclerotic complications triggered by cytotoxic oxysterols (Tesoriere and others 2015). On the other hand, in colorectal carcinoma (Caco-2) cells, indicaxanthin at 115 μmol/L exhibited a pro-apoptotic effect through the reactivation of promoter of an onco-suppressor gene resulting in accumulation of its protein product that controls the cell cycle (Naselli and others 2014).

Among the studies reviewed in this section there is certain inconsistency about the concentrations of betalains in cell culture assays. Although there is no toxicity associated with betalain consumption, growth of cell cultures may be inhibited at certain treatment concentrations. Indicaxanthin was considered to be cytotoxic to non-proliferating (endothelial) cells (Gentile and others 2004) and proliferating Caco-2 cells (Naselli and others 2014) at a concentration above 10 μmol/L, whereas the betaxanthin fraction was cytotoxic (IC_{50} 39 μmol/L) to proliferating hepatic carcinoma (HepG2) cells (Khan and others 2012).

Table 1–Cytotoxicity of betalains against various cell lines after 24-h exposure

Cell line	Pigment	Source	IC_{50}	Reference
Colon adeno-carcinoma cells (Caco-2)	Indicaxanthin	*Opuntia ficus-indica* fruit	115 μmol/L[a]	Naselli and others 2014
Human umbilical vein endothelial cells (HUVEC)	Indicaxanthin	*Opuntia ficus-indica* fruit	~15 μmol/L	Gentile and others 2004
	Betanin	*Opuntia ficus-indica* fruit	No cytotoxicity upto 50 μmol/L	
	Betanin/Isobetanin	*Beta vulgaris* L. root	35 μmol/L[a]	Nowacki and others 2015
Human lymphoma cells (Raji cells)	Betanin	*Beta vulgaris* L. root	~900 μmol/L	Kapadia and others 1996
Human melanoma cells (B16F10)	Betanin	*Hylocereus polyrhizus* fruit	~230 μmol/L[a]	Wu and others 2006
	Betanin/Isobetanin	*Beta vulgaris* L. root	25 μmol/L[a]	Nowacki and others 2015
Human chronic myeloid leukemia cells (K562)	Betanin	*Opuntia ficus-indica* fruit	40 μmol/L	Sreekanth and others 2007
Murine macrophage cells (RAW 264.7)	Gomphrenin I	*Basella alba* L. fruit	No cytotoxicity upto 100 μmol/L;	Lin and others 2010
Human hepatic carcinoma cells (HepG2)	Betaxanthins (partially purified)	*Rivina humilis* L. fruit	~39 μmol/L; ~6.5 μmol/L[a,c]	Khan and others 2012
	Betacyanins (partially purified)	*Rivina humilis* L. fruit	No cytotoxicity upto 58 μmol/L; ~31 μmol/L[a,d]	
	Betanin	n.a	200 μmol/L[b]	Krajka-Kuźniak and others 2013
	Betanin	*Beta vulgaris* L. root	~360 μmol/L[a]	Lee and others 2014
Non-tumour hepatocytes (THLE-2)	Betanin	n.a	> 200 μmol/L	Krajka-Kuźniak and others 2013
Human colonic adenocarcinoma cells (HT-29)	Betanin	*Beta vulgaris* L. root	< 25 μmol/L	Esatbeyoglu and others 2014a
	Betanin/Isobetanin	*Beta vulgaris* L. root	No cytotoxicity upto 40 μmol/L[a]	Nowacki and others 2015
Human liver hepatoma cells (Huh7)	Betanin	*Beta vulgaris* L. root	~25 μmol/L	Esatbeyoglu and others 2014a
Human breast cancer cell line (MCF-7)	Betanin/Isobetanin	*Beta vulgaris* L. root	25 μmol/L[a]	Nowacki and others 2015

[a]48 h exposure.
[b]72 h exposure.
[c]Molar concentration was calculated based on molecular weight of dopaxanthin, 390 g/mol.
[d]Molar concentration was calculated based on molecular weight of betanin, 550 g/mol.

Although the cytotoxic effect varies with cell type and duration of exposure (Table 1), these reports should be taken into consideration while deciding on dosages for assays involving indicaxanthin. Betacyanins (upto 100 μmol/L) are generally nontoxic when proliferating cell cultures are exposed to it for 24 h or longer (Gentile and others 2004; Lin and others 2010; Khan and others 2012; Nowacki and others 2015), in case of non-tumor cells (Krajka-Kuźniak and others 2013), but recently cyctotoxicity of betanin concentrations above 25 μmol/L was observed against hepatoma and adenoma cells (Krajka-Kuźniak and others 2013; Esatbeyoglu and others 2014a). Furthermore, toxicity of betacyanin fraction from *R. humilis* berry (IC_{50} 31 μmol/L) (Khan and others 2012) or betanin from red beet (IC_{50} 360 μmol/L (Lee and others 2014) or 200 μmol/L (Krajka-Kuźniak and others 2013) to HepG2 cell cultures has been observed after prolonged exposure. Recently, Gandía-Herrero and others (2014) have compiled a list of cell culture studies indicating the effect of betalains on various cell lines. A comprehensive list of cytotoxicity is presented in Table 1.

Betalains may play favorable physiological roles in fish species as well. The probable roles of dietary pigments, including anthocyanin, betalain, and carotenoids, were investigated in male *Colisa lalia* (flame-red dwarf gourami fish) with respect to body color, social behavior, and female preference (Baron and others 2008). Although betalain intake did not have effect on body color intensity, the color remained unchanged even after periods of social interaction. Unlike betalain-fed fishes, carotenoid- and anthocyanin-fed fish body color faded. This observation suggested that betalains may be involved in maintenance of body color.

From the literature reviewed for this section, it is clear that betalains exhibit a wide range of biological activities (a list of clinical efficacy studies is presented in Table 2), predictably because of their RSA (Esatbeyoglu and others 2014b). However, it is not clear which betalain at what concentration is effective for a particular physiological dysfunction or group of pathologies. As a major portion of the literature pertains to *in vitro* or cell culture studies, the effective concentration *in vivo* is yet to be ascertained. In the absence of a comprehensive study on various cellular mediators and corresponding gene expression vis-a-vis intracellular localization and stability and hepatic transformation of betalains, it will remain inconclusive as to how betalains protect cells and tissues from various physiological dysfunctions. However, recent evidence suggested that betanin—treated cell cultures showed translocation of nuclear factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) from cytosol to nucleus (Krajka-Kuźniak and others 2013; Esatbeyoglu and others 2014a). Nrf2 is a redox-sensitive transcription factor that modulates expression of genes coding for antioxidant and phase II enzymes central to endogenous antioxidant defence. In basic pH, Nrf2 is anchored to its inhibitor Keap-1 (Kelch-like ECH-associated protein-1) in cytosol. When oxidants accumulate, redox-sensitive cysteine residues of Keap-1 cause release of Nrf2, which subsequently translocates to the nucleus and bind to an antioxidant—responsive element (ARE) in the DNA. Betanin concentrations above 15 μmol/L can activate the Nrf2-dependent signal transduction pathway (Krajka-Kuźniak and others 2013; Esatbeyoglu and others 2014a). This pathway has also been implicated in anti-inflammatory response (Ruiz and others 2013), as listed in Table 2. Moreover, betanin induced an increase in the mRNA and

Table 2–Clinical efficacy of betalains from different plant sources against various disease models

Biological activity	Treatment (dose/frequency/ duration /route of administration)	Study model	Plant source	Reference
Cardioactive	4.9 mg betanin/NA/NA/i.v.	Rats	Beetroot	Krantz and others 1980
Anti-cancer	2.5 mg betanin/daily/ 20 wk/topical and oral application	Rat skins (papilloma), lungs (adenoma)	Beetroot	Kapadia and others 2003
	0.025–0.1 mg/mL betanin/daily/20 wk/oral drinking water	Mice (lung tumour)	Beetroot	Zhang and others 2013
Anti-inflammatory	2.7 mg betanin or 1.5 mg indicaxanthin/one time/ 16 h/culture medium	Human umbilical vein endothelial cell cultures	*Opuntia ficus-indica*	Gentile and others 2004
	55 mg gomphrenin/one time/ 24 h/culture medium	Murine macrophage cell cultures	*Basella alba*	Lin and others 2010
	1.5–7.5 mg indicaxanthin/one time /24 h/culture medium	Caco-2 cell cultures	*Opuntia ficus-indica*	Tesoriere and others 2014
	0.15–0.6 mg indicaxanthin/kg bw/6 times/40 h/oral gavage	Rats	*Opuntia ficus-indica*	Allegra and others 2014b
Hepatoprotective	10–150 μg/mL betanin/daily/ 2 mo/diet	Rats	Beetroot	Lee and others 2005
	6.3 mg betaxanthins and 12.7 mg betacyanins/kg bw/daily/ 4 wk/oral gavage	Male rats	Beetroot	Kujawska and others 2009
	6.3 mg betaxanthins and 12.7 mg betacyanins/kg bw/daily/ 4 wk/oral gavage	Lung and mammary glands of female rats	Beetroot	Szaefer and others 2014
Radioprotective	20 mg/kg bw/daily/33 d/oral gavage	Mice	Beetroot	Lu and others 2009
Neuroprotective	50–100 mg betanin/kg bw/daily/2 wk/oral gavage	Rat brains	*Portulaca oleracea*	Wang and Yang 2010
Diuretic	26 μg betanin and 110 μg indicaxanthin/kg bw/daily/1 wk/oral gavage	Rats	*Opuntia ficus-indica*	Bisson and others 2010
Hypolipidemic	30 mg betalains/3 times a day/1 d/oral	Human volunteers	Beetroot	Pietrzkowski and Thresher 2010
Osteoarthritis pain reliever	*ca.* 18 mg betalains/2 times per day/10 d/oral	Human volunteers	Beetroot	Pietrzkowski 2010
Anti-diabetic	9.6 mg betanidin/kg bw/daily/40 d/oral	Mice	*Hylocereus ocamponis*	Lugo-Radillo and others 2012
	25–100 mg betanin/kg bw/daily/60 d/oral	Rats	NA	Han and others 2015

NA, not available; i.v., intravenous; bw; body weight.

nuclear protein levels of Nrf2, and binding of Nrf2 to ARE sequences was followed by the phosphorylation of serine/threonine kinase (AKT), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) (Krajka-Kuźniak and others 2013). AKT, JNK, and ERK are involved in mitogen–activated protein kinase (MAPK) signaling pathway. Hence, betanin may activate MAPK pathway too. As of now, the following possible mechanisms are well supported by the observed changes in the cellular environment after administration of betalains:

(1) Owing to its RSA, betalains quench the radicals and cellular environment is relieved of the oxidative stress caused by toxicants, thereby restoring the redox balance.
(2) In addition to the above possibility, betanin may act as a signal *in vivo* to modulate gene expression through redox-sensitive transcription factors such as NF-κB, activator protein-1 (AP-1), Nrf2, and MAPK (Esatbeyoglu and others 2014b; Han and others 2015).
(3) In addition to redox-active transcription factors, indicaxanthin may carry out epigenetic modulation of gene expression to suppress tumor proliferation (Naselli and others 2014; La Rosa and others 2014).
(4) Betanin inhibits Maillard reaction *in vivo* preventing the formation of AGEs (Han and others 2015).
(5) There is a lack of information on the role of betalain transformation products, if any, *in vivo*.

Bioavailability

Betanin

Despite many odd studies on biological activity of betalains, we are still far away from deciphering the route of betalain absorption in the body. Bioavailability studies of betanin are compiled in Table 3 and 4. Findings from *in vitro* studies suggested that betanin absorption takes place in the intestine. When fed to rats, about 2.7% unmetabolized pigment was excreted each in urine and feces (Krantz and others 1980). It was assumed that most of the betanin ingested was degraded in the GI tract as indicated by digestibility studies. About 74%, 35%, and 60% betanin was metabolized in saline suspension of the stomach wall, small intestine, and colon, respectively, while liver perfusion experiments indicated that only

Table 3–Urinary excretion of betalains among human volunteers

Betalain	Intake (/kg bw)	Bioavailability (%)	Reference
Betanin	24.7 mg	2.7 ± 1.2 ($n = 8$)[a]	Krantz and others 1980
	~2 mg[b]	0.7 ± 0.2 ($n = 2$)	Kanner and others 2001
	~0.27 mg[b]	3.7 ± 0.2 ($n = 8$)	Tesoriere and others 2004
	~6.7 mg[c]	0.28 ± 0.08 ($n = 6$)	Frank and others 2005
Indicaxanthin	~0.5 mg[b]	76 ± 3.0 ($n = 8$)	Tesoriere and others 2004
	0.62 mg	21 ± 1.2 ($n = 15$)[a]	Allegra and others 2014b

Values are mean ± S.D. (n)
[a] Data collected in rats.
[b] Betanin or indicaxanthin equivalent in *Opuntia ficus-indica* fruit pulp.
[c] Betanin equivalent in red beet juice.

a small fraction of betanin was metabolized in the liver. Recovery in urine was confirmed by a later report (Reynoso and others 1999), but the degradation in stomach, small intestine, and colon was comparatively less, probably due to the nature of the sample used (betalain-rich extract). In addition, the study did not detect any metabolic effects of betalain extracts on hepatocytes when incubated for 7 h, signifying that the liver may not be the favorable site for betalain metabolism. Based on this pretext, researchers overlooked its metabolism in the liver, if any, thereby creating an information gap in comprehending betalains' bioavailability. To add to this, the analytical method may fail to detect betalain metabolic products. For instance, during spectrophotometric analysis of the recovered urine 24 h after feeding betalain extract, apart from intact betanin, no relevant metabolic product was detected (Krantz and others 1980). Similarly, among human volunteers, spectrophotometric analysis of betalains in urine revealed 0.5% to 0.9% bioavailability of betanin (Kanner and others 2001). After 2 to 4 h of ingestion, the pigment appeared in urine and it continued up to 12 h, but no metabolic product of betalain was detected. The absorption site was guessed to be the intestine by likening the process to quercetin glucoside absorption in the gut (Gee and others 1998). Tesoriere and others (2004) presented HPLC analyzed data on plasma and urinary pharmacokinetics of human volunteers fed 500 g cactus pear fruit pulp containing 16 mg betanin over a period of 12 h. Betanin appeared in plasma after 60 min and maximum plasma concentration (C_{max}) of 0.20 nmol/mL was reached in 3.1 h (T_{max}). The terminal elimination half-life ($T_{1/2}$) was 0.94 h for betanin. Lower $T_{1/2}$ for betanin, compared to indicaxanthin (Tesoriere and others 2004), suggested the possibility of lower distribution volume, higher clearance rate, or both. Betanidin was not detected in the study suggesting that deglucosylation was not required for betanin absorption. Variation in betanin's bioavailability in urine based on dietary source was apparent as betanin from cactus pear fruit was significantly more bioavailable (3.7%) (Tesoriere and others 2004) than that of red beet (Krantz and others 1980; Kanner and others 2001; Frank and others 2005). Spectrophotometer and LC-MS/MS analysis of urine collected from human volunteers fed red beet juice revealed only 0.28% betanin bioavailability (Frank and others 2005), which was comparable to that of anthocyanins (Manach and others 2005). These values, especially the bioavailability and $T_{1/2}$ (7.43 h) (Frank and others 2005) were inconsistent with plasma pharmacokinetics data (Table 4). Since Frank and others (2005) did not report on plasma pharmacokinetics, the variation in data could not be precisely accounted for. In addition, metabolic transformation products of betalains, if any, were not considered, although some unidentified peaks were observed in the HPLC profile of the urine samples (Frank and others 2005). Thus, metabolic transformation products of betalains were eluding detection by researchers and, hence, bioavailability studies so far are not inclusive. Moreover, bioavailability studies cited in this section appear to have discrepancies in one or more determinants of the experiments that should be taken care of, such as sampling duration, processing of sample, analytical method, and subsequent quantification of betalains. Considering betalains' poor stability (Khan 2015), variation in sampling duration and processing of sample may affect the results. Inconsistent results could also be partially attributed to different analytical methods that require absorbance at 535 to 538 nm and subsequent quantification using corresponding extinction co-efficients, 65,000 L/mol.cm (Kanner and others 2001; Tesoriere and others 2004) and 60,000 L/mol.cm (Frank and others 2005). The cumulative effect of the variations in the experiments may be responsible for the inconsistency in bioavailability data for betanin, especially from red beet juice (Kanner and others 2001; Frank and others 2005). Nevertheless, it is undeniable that betanin's bioavailability is low (Table 3 and 4). In an attempt to understand the reason for limited bioavailability of betanin, trans-epithelial transport of betanin was studied in Caco-2 cells (Tesoriere and others 2013b). The results provided further evidence on betanin absorption through intestinal epithelial cells without any metabolic transformations. Red beet betanin was less absorbed compared to that of cactus pear, probably due to matrix effect. Betanin exhibited a permeability coefficient which showed significant bidirectional values and nonlinear efflux kinetics. This and other evidence suggested that betanin absorption was selectively hindered by a multidrug resistance-associated protein 2 (MRP2)-mediated efflux as well as the food matrix. On the other hand, poor bioavailability of betanin could also be attributed to degradation of the pigment in the GI tract and/or postabsorptive distribution in the body. Regarding degradation of betanin in the GI tract, digestibility studies involving dissected pieces of small intestine showed that 26% to 60% of betalains, especially betacyanins or betanin, were significantly metabolized indicating a possible reason for poor bioavailability (Krantz and others 1980; Reynoso and others 1999). Similarly, *in vitro* simulation studies of betanin degradation at oral, gastric, and small intestinal phases revealed about 50% pigment loss (Tesoriere and others 2008). Moreover, betanin bioaccessibility was limited by food matrix, which could be a reason for low, as well as differential, bioavailability depending on dietary source. Furthermore, the low bioavailability could also be attributed to postabsorptive distribution of betanin in different body compartments such as erythrocytes (Tesoriere and others 2005).

Indicaxanthin

Indicaxanthin's bioavailability has been reported to be much more than that of betanin in human volunteers, as well as, rats as evident from urine and plasma pharmacokinetics data (Table 3 and 4). Among human volunteers who ingested 500 g cactus pear fruit pulp containing 28 mg indicaxanthin, HPLC analysis of their urine could detect 76% of ingested indicaxanthin (Tesoriere and others 2004). Also, the molar ratio of the excreted betalains was comparable to the ratio of the $AUC_{0–12}$ of betanin and indicaxanthin (Tesoriere and others 2004). This could be because of similar metabolic pathways of indicaxanthin and betanin. In plasma, indicaxanthin was detected after 60 min and C_{max} was 6.9 nmol/mL. The T_{max}, and $T_{1/2}$ were 3.0 h, and 2.36 h.

Table 4–Plasma pharmacokinetics after betalain consumption

Betalain	Intake (/kg bw)	Approx. time taken to appear *in vivo* (min)	C_{max} (nmol/mL)	T_{max} (h)	$T_{1/2}$ (h)	Subject	Reference
Betanin	~0.27 mg[a]	60	0.20 ± 0.02	3.10 ± 0.25	0.94 ± 0.07	Human volunteers ($n = 8$)	Tesoriere and others 2004
Indicaxanthin	~0.5 mg[a]	60	6.9 ± 0.54	3.0 ± 0.27	2.36 ± 0.17	Human volunteers ($n = 8$)	Tesoriere and others 2004
	0.62 mg	45	0.22 ± 0.02	2	1.15 ± 0.11	Rats ($n = 15$)	Allegra and others 2014b

Values are mean ± S.D.
[a] Betanin or indicaxanthin equivalent in *Opuntia ficus-indica* fruit pulp.

Indicaxanthin's high bioavailability was supported by a report on pharmacokinetic study with a single oral administration of 2 μmol/L indicaxanthin/kg body weight to rats (Allegra and others 2014b). This study reported plasma concentration of indicaxanthin at 0.22 μmol/L ($n = 15$), with a $T_{1/2}$ of 1.15 h, which was almost half of the value reported previously in human volunteers (Tesoriere and others 2004). Furthermore, bioavailability of indicaxanthin in rat urine was only 21% (Allegra and others 2014b) against 76% in human volunteers (Tesoriere and others 2004). The difference in the plasma C_{max} and renal excretion in human and rat studies may be due to obvious differences in the rat and human systems as observed for phenolic compounds (Crozier and others 2009). Not only this, the reason could also be the source of indicaxanthin: fresh fruit pulp (Tesoriere and others 2004) and purified indicaxanthin (Allegra and others 2014b), which may be more prone to oxidation in the GI tract. The inference from these studies on indicaxanthin bioavailability is that the purified compound is relatively less bioavailable. However, none of the studies quantified any betalain metabolic products assuming that only a small fraction of the absorbed betalains is metabolized in the liver, as suggested by earlier reports (Krantz and others 1980; Reynoso and others 1999).

Although indicaxanthin absorption via intestinal epithelial cells was earlier suggested, it was demonstrated in Caco-2 cells (Tesoriere and others 2013b). Further, in an attempt to measure the factors determining indicaxanthin diffusion, H^+-dependent carriers, efflux transporters, pigment concentration, and permeability coefficient were studied (Tesoriere and others 2013b). The study ruled out involvement of carriers and transporters in indicaxanthin influx or efflux, while pigment concentration and permeability coefficient had a linear effect on the influx. Apparent permeability coefficient of the diffusion was non-polarized, time and concentration dependent, and unaffected by inhibitors of membrane transporters.

The percentage of indicaxanthin that is not bioavailable in urine or plasma might undergo various processes including postabsorptive distribution to different parts of body or degradation in the GI tract. Absorption of indicaxanthin in erythrocytes has been reported (Tesoriere and others 2005), perhaps, through paracellular diffusion, independent of carriers (Tesoriere and others 2013b), as in the case of phenolic acids (Lafay and Gil-Izquierdo 2008). In the GI tract, it appears that indicaxanthin is almost able to escape the degradation process. *In vitro* simulation studies comprising oral, gastric, and small intestinal phases revealed that only a minor quantity of indicaxanthin was lost through all the digestive steps (Tesoriere and others 2008). In addition, indicaxanthin was wholly bioaccessible and it was not affected by food matrix. This and the unidirectional concentration-dependent permeability coefficient reasonably explained indicaxanthin's high bioavailability. However, claim on indicaxanthin's complete evasion of the digestive steps (Tesoriere and others 2008) needs further verification as earlier digestibility experiments involving dissected pieces of stomach, small intestine, and large intestine suggested significant degradation of betalains in the GI tract (Krantz and others 1980; Reynoso and others 1999).

Metabolic transformation products

Most of the studies on urinary pharmacokinetics of betanin point to low renal excretion. However, without accounting for the metabolic products, the data on bioavailability of betalains is incomplete, because recent case studies have revealed the possibility of metabolic transformation (Cserni and Kocsis 2008; Roemmelt and others 2014). Autopsy of a cancer patient, who used to consume large quantities of red beet as an adjunct treatment, revealed a case of purple colon (Cserni and Kocsis 2008). After excluding all the possible causes of the colon discoloration, it was concluded that the purple color was most likely beetroot pigment, which disappeared on fixing for histological sectioning. Without analytical confirmation, it was assumed that post-mortem changes might have led to diffusion of the pigment from the lumen of the colon to the serosal surface because such discoloration was not found *in vivo*. While solving a similar case of a person, who apparently died (the reason for death was not explained) a few hours after consuming beetroot, it was analytically confirmed that beetroot pigments were responsible for colon discoloration (Roemmelt and others 2014). The color was extracted from the discolored colon, and analyzed using LC-HRMS. In the sample, chemists could detect betanidin, betanin, and isobetanin. In addition to betanin, its 2 phase II metabolites, betanidin glucuronide (m/z 565.1294) and betanidin sulfate (m/z 469.0541), were identified for the first time in urine based on the characteristic ion fragmentation pattern. In the light of recent data on plant secondary metabolite transformation products playing an active role in signaling and modulation of gene expression, in addition to RSA (Crozier and others 2009), it is imperative to take up a holistic experimental approach to study bioavailability of betalains. Consideration should be given to factors affecting bioavailability of phytochemicals, such as dietary source and food matrix, instability of their molecules in the digestive environment, bacterial degradation in the gut, and mechanism of absorption, as well as processing parameters such as cooking, postharvest processing, stabilizers, and preservatives (Baur and Sinclair 2006; Crozier and others 2009; Kamiloglu and others 2013). Interestingly, betalain bioavailability may be improved by stabilization through complex formation with antioxidant metals such as selenium. Betalain-Se complex formation may be possible in the presence of a biological reducing agent such as ascorbic acid, which also can stabilize betalains (Khan and Giridhar 2014). It may be hypothesized that such complexes are absorbed via a Se absorption route, because organic Se is better absorbed than free inorganic Se. Hence, betalains' bioavailability needs to be systematically assessed in a model food, as well as in a realistic food matrix, to understand, apart from the renal route, the other pathways of betalain elimination, if any, such as biliary excretion or enterohepatic circulation and metabolism.

Table 5–Bioavailability of different phenolic compounds that exhibit various biological activities

Phenolic compound	Administered form/dose/frequency/ duration of treatment	C_{max} (μmol/L)	T_{max} (h)	Urinary excretion in 24 h (% ingested dose)	Reference
Caffeic acid	Purified/500 mg/once a day/1 d	ND	ND	10.7 ± 2	Olthof and others 2001
	Red wine/1.8–2.7 mg/once a day/1 d	0.02–0.03	0.5–1	ND	Simonetti and others 2001
Ferulic acid	Breakfast cereals/260 mg/once a day/1 d	0.15–0.21	1–3	3.1 ± 1.3	Kern and others 2003
Gallic acid	Purified/50 mg/once a day/1 d	4.6 ± 0.41	1.3–1.5	36.4 ± 4.5	Shahrzad and others 2001
	Assam black tea/50 mg/once a day/1 d	4.7 ± 0.5	1.4–1.5	39.6 ± 5.1	
Quercetin glucosides	Fried onions/~140 mg flavonols/oral/single dose/1 d	0.48 ± 0.25	0.5–3	4.7 ± 0.42	Crozier and others 2009
Isoflavones	Baked soybean flour/~32 mg/oral/single dose/1 d	2.22 ± 0.26	4.7–5.4	29.5 ± 13.6[a], 72.1 ± 16.4[b]	Hosoda and others 2011
Resveratrol	Radiolabeled/25 mg/oral/single dose/1 d	2 ± 0.16	~1	16–17	Walle 2011
Anthocyanins	NA	0.015–0.06	0.75–1	0.3–0.6	Manach and others 2005

NA, not applicable.
[a]Genistin and its metabolites.
[b]Diadzin and its metabolites.

Based on the available literature, it may be said that the physiological potential of betanin, which is relatively more stable at a higher pH, is a strong radical scavenger and is reasonably bioactive, has been severely curtailed by its low bioavailability (0.28% to 3.7%) (Table 3), which is comparable to that of dietary phenolics (Table 5). Phenolic acids, such as cinnamic acid and benzoic acid derivatives, are the main polyphenols in the human diet, and they are bioavailable. The aglucone forms of these compounds mostly get absorbed in the stomach (caffeic, ferulic, gallic acids) or small intestine, probably, through monocarboxylic acid transporters (MCTs) or paracellular diffusion (Lafay and Gil-Izquierdo 2008). Esterified forms of phenolic acids show reduced bioavailability compared to small and simple acids (Lafay and Gil-Izquierdo 2008). Similarly, as reviewed by Del Rio and others (2013), other polyphenols such as flavonoid glucosides release aglucones following cleavage by lactase phloridzin hydrolase (LPH; an enzyme with broad substrate specificity for flavonoid-*O*-β-D-glucosides) in the brush border of epithelial cells in the small intestine. These aglucones are then absorbed via the apical membrane by passive diffusion. Flavonoid glucosides that enter the small intestine intact are hydrolyzed by cytosolic β-glucosidase (CBG) within the epithelial cells. It was proposed that through glucose transporter GLUT2, adenosine triphosphate-binding cassette (ABC) family of transporters, including multidrug resistance protein (MRP) and P-glycoprotein mediated efflux, a fraction of the metabolites exits the epithelial cells into the lumen of the small intestine (Del Rio and others 2013). This leads to a decrease in the bioavailability of these compounds. In contrast to most (poly)phenols, in the case of isoflavones, glucosylation plays an important role in enhancing its bioavailability by facilitating active transport, reduced interaction in the food matrix, better solubility near the intestinal epithelial cells, and protection from gut bacteria by the sugar moiety (Vitale and others 2013). Although there are contrasting reports, it is most likely that LPH action releases aglucones which are absorbed faster from the lumen of the small intestine, compared to glucosides (Vitale and others 2013). Similarly, there is a need for investigating the bioavailability of various betalain forms including aglucones, diglucosides, and their derivatives, to document a pattern if there is one.

The (poly)phenolic compounds which closely resemble betalains, in terms of structure and function, are anthocyanins and stilbenes (for example, resveratrol). Very small fractions of ingested anthocyanins appear to be rapidly absorbed and eliminated. This could be explained through tissue distribution (bladder, prostate, testes, heart, and adipose tissue), effect of pH (positively charged flavylium in the stomach and carbinol pseudobase in the small intestine), activity of digestive enzymes, biliary acids, and the colonic microbiota, and also the food matrix (Fernandes and others 2014). The sensitivity of the analytical method, the type of sugar moiety attached, and the anthocyanidin structure influence anthocyanins' absorption and excretion, which is further complicated by the spectrum of structurally diverse anthocyanin pigments present in a sample. This leads to difficulty in quantification and linking of the trace levels of complex anthocyanins in plasma and urine to absorption, hepatic transformation, and excretion after 3′-*O*-methylation, which can transform cyanidin to peonidin, and delphinidin to petunidin, whereas 5′-*O*-methylation of petunidin produces malvidin (reviewed by Del Rio and others 2013). Besides, some anthocyanin structures, such as 3′-hydroxyanthocyanin and pelargonidin-3-*O*-glucoside, are more readily absorbed compared to cyanidin-3-*O*-glucoside and 3′,4′-dihydroxy analog of the former. This means that substitution at the 3′- and 4′-positions affects the uptake (Del Rio and others 2013). Apart from anthocyanins, the structural similarity of betalains with resveratrol has been highlighted, thereby exhibiting comparative biological activities (Gandía-Herrero and others 2014). Resveratrols as such are poorly bioavailable, but methylated resveratrols, which are resveratrol analogs, are better bioavailable (Walle 2011). Taking cue from enhanced bioavailability of methylated resveratrol (Walle 2011) and isoflavone complex (Lee and others 2007), relatively more stable selenium–betalain complex (Khan and Giridhar 2014), silicon–betalain complex (Molina and others 2014), and other stabilized betalain formulations (Khan 2015) could be explored for enhanced biolavailability.

In several of the studies reviewed above, *in vitro* antioxidant and biological activities of betalains have been suggested to be more efficient than ascorbic acid, resveratrol, and phenolic acids. Since ascorbic acid's bioavailability ranges from 50% to 100% depending on the dose (Davey and others 2000), *in vivo* biological activity also will be more pronounced, while, owing to poor absorption, it is unlikely that the *in vitro* effects of betalains will be proportionally exhibited *in vivo*. Although various pharmacological activities of betalains (Table 3) have been observed *in vivo*, the likely optimum concentration for daily intake may only be decided after systematically addressing poor stability and bioavailability issues.

Conclusions

This review presents the relevant literature sources to credit betalains' ability to scavenge free radical *in vitro* and *in vivo* for its perceived bioactivity. The studies pertaining to mechanistic details of antioxidant activity of betalains are discussed to establish a basis for the clinical efficacy of betalains. Moreover, based on the literature

sources discussed in this review, it is established that betalain preparations from many plant parts are safe to consume. However, there is a lack of data on the safety of the betalains processed by various technologies. This review presents the prospective observational and clinical efficacy studies to critically analyze the effective concentrations of betalains. Despite having a voluminous literature on betalains' nutrient value, production, processing, value-addition, food, pharmaceutical, and cosmetic industries have not benefited proportionally from the research findings. This is because of a lack of synthesis of the available literature data specially related to safety, clinical efficacy, and bioavailability to come up with tangible experimental approaches. The initial enthusiasm among researchers has abated rapidly owing to the poor stability and bioavailability of betalains, except for indicaxanthin. In general, betacyanins are absorbed less, due to glucosylation, which necessitates these pigments to compete with dietary sugars and then, a portion of the absorbed betanin is pumped out of the cells. In contrast, betaxanthins may readily diffuse across cell membrane unidirectionally. Since betaxanthins are prone to degradation, future research should be focused on technologies to enhance their stability *in vitro* and in gastric environment, and bioavailability by employing a blend of conventional (such as encapsulation and complexation) and emerging techniques (such as nanotechnology for targeted delivery and increased solubility and stability in a wide range of formulations) for structural modification of betalain pigments. Nevertheless, stabilization may compromise the bioavailability, as in the case of glucosylation which stabilizes pigments like betanin and gomphrenin, but reduces bioavailability and RSA. To solve this conundrum, there is much need for collaboration among food scientists, nutritional biochemists, and chemists to generate data on structural modifications to increase stability and bioavailability, and on mechanisms of biological activities, thereby increasing usages.

There is lack of data on the bioavailability of betalains. The available literature is inconsistent about bioavailability (%) and plasma kinetics, partly because the reported studies have not considered tissue distribution, if there is any, and metabolic transformation products of betalains. The inconsistency might have also stemmed from poor stability and high solubility in water which thwarts sample processing. Also, the method of quantification of betalains in bioavailability studies needs an update. For practical relevance, stabilized betalains such as encapsulated purified betalains could be used for bioavailability studies in the future. This will also expedite efforts to commercialize the technology by carrying out studies with uniform concentration for consistent results. Considering the earlier suggested concentration, biological activity and bioavailability of betalains, the concentration required for coloring foods and for effective biological activity should be such that the daily intake of betalains should not exceed 100 mg betanin and 50 mg indicaxanthin in their purified forms (encapsulated, preferably). To bring a consensus to this, more studies are required involving health professionals.

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