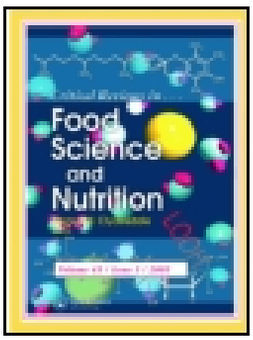

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Betalains, the nature-inspired pigments, in health and diseases

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Betalains, the nature-inspired pigments, in health and diseases

Abstract:

Betalains are unique nitrogen-containing pigments found exclusively in families of the Caryophyllales order and some higher order fungi, where they replace anthocyanin pigments. Betalains, consisting of betacyanins and betaxanthins are generally used as color additives in food. This review discusses on the favorable effects of acute and chronic consumption of betalains, whose edible sources consist primarily of red beetroots (*Beta vulgaris*) and prickly pears (fruit of the Opuntia genus of cacti). Moreover, it encompasses *in vivo* and *in vitro* studies about the bioavailability and bioaccessibility of betanin and indicaxanthin. It seems that treatment with betalains and betalain-rich diets is not only non-toxic but could also prove to be a promising alternative to supplement therapies in oxidative stress-, inflammation-, and dyslipidemia-related diseases such as stenosis of the arteries, atherosclerosis, hypertension, and cancer, among others. Due to its toxicological safety, accessibility, low price, biodegradability, and potentially advantageous biological effects on health, the incorporation of betalains in food manufacturing and related industries could pave the way to overcome current concerns over the health risks of artificial colors. Nevertheless, further studies using pure betalains are required to gain a deeper understanding of their precise biological functions.

Keywords

prickly pear, red beetroot, betanin, indicaxanthin, *in vivo*, *in vitro*

Abbreviations: **ALT:** alanine aminotransferase, **AST:** aspartate aminotransferase, **BALF:** broncho alveolar lavage fluid, **BFV:** blood flow velocity, **BMI:** body mass index, **BP:** blood pressure, **CAT:** catalase, **CCL_4:** carbon tetrachloride, **CD:** conjugated diene, **COX-2:** cyclooxygenase-2, **CRB:** cooked red beetroot, **cumOOH:** cumene hydroperoxide, **DBP:** diastolic blood pressure, **DMBA:** 7, 12-dimethylbenz[a]anthracene, **DNMT:** DNA methyl transferases, **EDTA:** Ethylenediaminetetraacetic acid, **eNOS:** endothelial nitric oxide synthase, **FMD:** flow-mediated dilation, **GOD:** glucose oxidase, **GPx:** glutathione peroxidase, **GR:** glutathione reductase, **GSH:** glutathione, **GSTs:** glutathione S-transferases (**GSTA:** class Alpha, **GSTM:** class Mu, **GSTP:** class Pi, **GSTT:** class Theta), **Hcy:** homocysteine, **HDL:** high-density lipoprotein, **hsCRP:** high-sensitivity C-reactive protein, **HUVEC:** human umbilical vein endothelial cell, **ICAM-1:** interacellular adhesion molecule 1, **IL:** interleukin, **iNOS:** inducible nitric oxide synthase, **i.v.:** intra venous, **/kg $_{b.w}$** = /kg $_{body\ weight}$, **LDL:** low-density lipoprotein, **LDL-c:** low-density lipoprotein-cholesterol, **LPS:** lipopolysaccharides, **MDA:** malondialdehyde, **MPO:** myeloperoxidase, **MRP2:** multidrug resistance-associated protein 2, **NDEA:** N-nitroso diethylamine, **NF- κB:** nuclear factor-κB, NMBA: N-nitroso methyl benzyl amine, **NMDAR:** N-methyl-D-aspartate receptor, **NO:** nitric oxide, **non-HDL-c:** non-high-density lipoprotein-cholesterol, **NOX:** NADPH oxidase, **NQO1:** NAD(P)H quinone dehydrogenase 1, ***OFI:*** *Opuntia ficus-indica*, **OGTT:** oral glucose tolerance test, **PAD:** peripheral artery disease, **PBMCs:** peripheral blood mononuclear cells, **PCV:** polycythemia vera, **PG:** prostaglandin, **PGE_2:** prostaglandin E_2, **PMA:** phorbol 12-myristate 13-acetate, **PORH:** post-occlusive forearm skin reactive hyperaemia, **PPF:** prickly pear fruit, **PPFJ:** prickly pear fruit juice, **RBR:** red beetroot, **RBJ:** red beetroot juice, **RH:** reactive hyperemia, **SOD:** superoxide dismutase, **TAGs:** triacylglycerols, **TC:** total cholesterol, **SBP:** systolic blood pressure, **SHFAs:** short-chain fatty acids, **tHcy:** total homocysteine, **TNFα:** tumor necrosis factor alpha, **VCAM-1:** vascular cell adhesion molecule-1, **WBC:** white blood cells

Introduction:

Betalains are water soluble, indole-derived natural colorants composed of a nitrogenous core structure, named betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid]. Condensation of betalamic acid with imino compounds or amino acids/derivatives forms a variety of red-violet betacyanins (mainly betanin) or yellow-orange betaxanthins (mainly indicaxanthin and vulgaxanthin), respectively (Belhadj Slimen, Najar, and Abderrabba. 2017; Khan. 2016).

In contrast with widely distributed anthocyanins in our diet, betalains was mainly found in Beets and Opuntia cactus fruits. The occurrence of betalains and anthocyanins is mutually exclusive.

Concern over the toxicological safety and the rigorous and costly toxicological testing of synthetic dyes, on the one hand, and valuable properties of betalains such as their safety, abundance, easily extractable, bioaccessibility and biodegradability on the other hand, have encouraged the development and application of natural pigments such as betalains as food ingredients. Based on Title 21 of the Code of Federal Regulations part 73.40 of the Food and Drug Administration (FDA) in the USA, beet powder with EEC No. E 162, is considered a permitted colorant, which today, is used to color a variety of fabric, foods, pharmaceuticals etc. with compatible physicochemical properties as well as to establish dye-sensitized solar cells (Richhariya et al. 2017).

This is the first review ever discussed on cell, animal and human studies about betalains and theirs main edible sources (Stintzing et al. 2008), red beetroot (RBR) and prickly pears fruits (PPFs). Moreover, all the main inclusion criteria, sample size, randomization, blinding, assignment, intervention groups, study design and methods of studies have been listed in three tables according to their study type. To this end, the authors have searched through English language papers and registered clinical trials in Scholar Google, Pubmed, Science Direct, Elsevier, Nature, Proquest, Springer & Kluwer, and Taylor & Francies database, and the international

clinical trials registry portal website from the inception to January 2018. Reports support the view that betalains not only pose no threat to human and the environment, but also, have beneficial effects on oxidative stress, lipid peroxidation, inflammatory status, cytotoxicity, apoptosis of tumour cells, Hcy concentration, blood pressure, flow-mediated dilation (FMD), endothelial dysfunction, bioelectric activity on glutamatergic synapses and antispasmodic effects on the intestinal motility. Therefore, authors hope that this review gives researchers and authorities a comprehensive view of exciting properties of betalains, to make the right decisions for their future studies and more applications of betalains in the food, cosmetic and pharmaceutical industries.

Bioavailability, bioaccessibility, and transport:

In a rat study, after i.v. injection of 4.5 µmol betanin, the mean half-life of betanin in plasma was 32 min and the urine was colored red after less than 3 min. This indicates that excretion of betanin occurs rapidly. Betanin urinary excretion was 88.0 + 6.7% of the injected dose after 4 h. But 24 h after the oral administration of 4.5 µmol betanin, it could not be detected in the blood, however, 3% was recovered in the urine and 3% in the feces. Also, biliary excretion was not a major rout of elimination and only 1.1±0.4% of the added betanin to the isolated perfused liver was eliminated in the bileand most of them was recovered from the perfusion fluid, after 3h. Furthermore, betanin was largely metabolized by the tissues lining the gastrointestinal tract, and orally-administered betanin was poorly absorbed perhaps due to the extensive metabolism of the pigment in the walls of the gastrointestinal tract. However, there were great variations between the individual tissue samples in their capacity to metabolize betanin. In this study, the stomach wall, small intestine and the colon metabolized about 75, 35 and 60% of the added betanin, respectively (Krantz, Monier, and Wahlström. 1980).

In an inflammation model of rats who orally consumed 2 µmol indicaxanthin/kg $_{b.w.}$, maximum plasma concentration and half-life of indicaxanthin were 0.22 ± 0.02 µmol/l and 1.15 ± 0.11 h, respectively (Allegra et al. 2014b).

A human study on the healthy participants shows that after supplementation with a single dose of a commercial red beetroot juice (RBJ), the amount of intact betalains (betanin and isobetanin) recovered in urine was 1001±273µg, corresponding to 0.28±0.08% of total administered dose, and maximum excretion rate, which was observed after 3.0 h (range= 2.5–8.0 h), was 91.7± 30.1 µg/h. The terminal elimination rate constant (λ_z), the corresponding half-life, and the expected total betalain amount excreted in the urinewere 0.097±0.021 h^{-1}, 7.43±1.47 h, and 1228±291µg, respectively. The urinary excretion rates of un-metabolized betalains were fast and appeared to be mono-exponential suggesting a one-compartment model. Therefore, the authors believed that the bioavailability of betalains was low because the minor portion of the systemic elimination is due to the renal clearance. Furthermore, they suggest that the measurement of unchanged compounds and their metabolites in plasma, urine, and bile is necessary to investigate betalain bioavailability (Frank et al. 2005). Another human study shows that, after consumption of a single dose of PPF pulp by healthy participants, betanin and indicaxanthin reached their maximum plasma concentrations after 3 h, and declined according to first-order kinetics. The half-life of betanin (0.94 ± 0.07 h) was shorter than that of indicaxanthin (2.36 ± 0.17 h), and both compounds had been disappeared from the plasma 12 h after intake. Over 12 h, the urinary excretion of indicaxanthin and betanin was 76 ± 3.0% and 3.7 ± 0.2% of total ingested betalain content, respectively (Tesoriere et al. 2004a).

However, Clifford et al., could not detect betanin in the plasma of healthy males after consumption of a single dose of RBJ and cooked red beetroot (CRB) (Clifford et al. 2016). In the other study, fractionated extracts of high-pigment strains of RBR, containing

vulgaxanthins I and II, and (iso) betanin, were added into rodents diet for betalains bioavailability assessment. This diet had no statistical favorable effecton the tissue levels of phase II enzymes (Lee et al. 2005).

In an in vitro study on the simulated gastrointestinal digestion, loss of betacyanins, either purified or food-derived, was observed during the small intestinal phase of digestion. Food matrix could prevent betanin and Isobetanin from decay at the gastric-like environment, but, did not influence the minor loss of indicaxanthin at the gastric step of digestion and vulgaxanthin-I is reduced through all digestion steps. Unlike consumption of betalain-rich food, ingestion of pure pigments leads to accumulation of more betalamic acid due to the degradation of pure betanin in the gastrointestinal tract. After ultracentrifugation of the post-intestinal digesta betaxanthins were wholly soluble in the aqueous fraction whereas release of betacyanins from the food matrix was incomplete. Therefore, the bioaccessibility of betaxanthins was higher than betacyanins. Bioaccessibility of dietary betaxanthin is controlled by its stability against digestion. Whereas, the other factors such as the food matrix and processing style could affect the betacyanin bioaccessibility. It seems that betalains bioaccessibility of raw RBR per serving size (68 g) is more than PPF (149 g). iIn Caco-2 cells monolayers seeded on Transwell® insert, a well-established model of the intestinal barrier, apparent permeability coefficients (P_{app}) in the absorptive direction was $(3.2 \pm 0.3) \times 10^{-6}$ cm s^{-1} and $(4.4 \pm 0.4) \times 10^{-6}$ cm s^{-1} for betanin and indicaxanthin respectively. After treatment of Caco-2 cell monolayers with EDTA, which affects paracellular permeability via loosening of the tight junctions, permeation of both indicaxanthin and betanin increased significantly and underwent no metabolic transformation. Bidirectional, trans-epithelial transport of indicaxanthin was non-polarized, linear as a function of concentration and time, and not affected by membrane transporter inhibitors. Whereas, bidirectional P_{app} value of betanin was significantly different. It had non-linear efflux kinetics. A kinetic model including non-saturable (K_d = 0.042 µLcm^{-2} min^{-1}) and saturable segment such as the apical multidrug resistance-

associated protein 2 (MRP2; K_m = 275 µM; J_{max} = 42 pmol min^{-1} cm^{-2}) may describe the concentration-dependent efflux of betanin. In addition, food matrix (RBR more than PPF) decreases the rate of betanin absorption, but, had no effect on indicaxanthin trans-epithelial transfer through a simulated gastrointestinal digestion of betalainic food. Authors conclude that dietary betanin and indicaxanthin can be absorbed mainly by intestinal epithelial cells through paracellular junctions, Furthermore, the other trans-membrane permeation system must be considered for betanin, because its absorption is limited by a MRP2-mediated efflux and negatively affected by food matrix. In conclusion, bioavailability of indicaxanthin in our body is higher than betanin (Tesoriere et al. 2013b). In an in vitro simulated conditions of the gastrointestinal tract, betalains were relatively stable, as their radical scavenging activity decrease from 75% inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH•) to about 38%. Acid and bile salt tolerance tests showed that pH below 3 and concentrations of the bile salts up to 4% have no great influence on stability of betalains. (Pavlov et al. 2005).

After the transition of betalains through the gastrointestinal tract and absorption via enteral epithelial cells, they enter the bloodstream and gain the ability to incorporate into RBCs (red blood cells) and lipoproteins.

Consumption of betalainic food or incubation of isolated RBCs with dietary betalains incorporate betalains into human RBCs. The concentration of indicaxanthin in isolated human RBCs at 1, 3, 5 and 8 h post-supplementation with PPF was undetectable, at maximum (1.03± 0.2 µM), about half, and at even smaller amounts, respectively. However, Betanin (30.0±5.2 nM) was found only at 3h (Tesoriere et al. 2005). Incubation of plasma with betanin and betaxanthin led to the incorporation of them into LDL (max binding for betanin= 0.52 ± 0.08 and for indicaxanthin= 0.51 ± 0.06 nmol/mg $_{\text{LDL protein}}$) (Tesoriere et al. 2003). Also in a human study, LDL

was isolated 3 and 5 h after consumption of the PPF meal incorporated with betalains at concentrations of 100.5 ± 11 and 50 ± 7.2 pmol/mg $_{\text{LDL protein}}$, respectively (Tesoriere et al. 2004a).

In a rat study, administered indicaxanthin (in an amount compatible with a dietary consumption of PPF in humans) crossed from the rat blood-brain barrier and accumulated in the brain. Kinetic measurements showed that after 1h from oral administration of 2 µmol indicaxanthin/kg $_{\text{b.w.}}$, indicaxanthin appeared in the rat brain, and after 2.5 h, its peak (20 ± 2.4 ng indicaxanthin per the whole brain) was detected. This molecule showed the first-order kinetics within 4 h (Allegra et al. 2015).

Briefly, food matrix, processing method, and digestive stability can affect the bioaccessibility of betalains. It seems that most of the betanin consumed is metabolized and degraded in the gastrointestinal system. However, the plasma half-life and post-intestinal bioaccessibility of indicaxanthin are higher than betanin. Furthermore, indicaxanthin can pass through blood brain barrier (BBB), but, there is no study yet about the permeation of betanin from BBB and its appearance in the brain.

Antioxidant effects of Betalains

Betalains belong to the class of dietary cationized antioxidants (Kanner, Harel, and Granit. 2001). Betanin and its aglycone counterpart, have lipoperoxyl radical scavenging and antioxidative effects (Livrea and Tesoriere. 2013).

Many studies showed that these phytochemicals can act through several antioxidant defense mechanisms of the body and improve redox balance to overcome oxidative stress damages. In a cell study, betanin dose-dependently scavenged DPPH-, galvinoxyl-, superoxide-, and hydroxyl-radicals and induced Nrf2 transcription factor and resulted in an elevation of heme oxygenase-1 (HO-1)

protein levels, paraoxonase-1 (PON-1) transactivation and cellular glutathione (GSH). So betanin act via two main mechanisms; 1) induction of antioxidant defense mechanism, and 2) free radical scavenging mechanism (Esatbeyoglu et al. 2014). Treatment with betanin (2–500 μM) significantly inhibits reactive oxygen species (ROS) production in neutrophils (by 15–46%, depending on the ROS detection assay), and in the stimulated and unstimulated neutrophils, reduces and increases the percentage of DNA in comet tails, respectively, only after 24 h of treatment (Zielińska-Przyjemska et al. 2012). In THLE-2 and HepG2 cell lines, Nrf2 is translocated from the cytosol to the nucleus via betanin treatment (2, 10 and 20 μM). But only in the non-tumoric THLE-2 cells, not in tumoric HepG2 cells, elevation in the levels of mRNA and nuclear protein of Nrf2 and its binding to ARE sequences accompanied with the phosphorylation of c-Jun N-terminal kinase (JNK), serine/threonine kinase (AKT), and extracellular signal-regulated kinase (ERK) were detected. Also, in THLE-2 cells, the mRNA and protein levels of Glutathione S-transferases (GSTs) classes such as GST-P_i, GST-mu, GST-Theta, and NAD(P)H quinone dehydrogenase 1 (NQO1) significantly increased (Krajka-Kuźniak et al. 2013). ~~But betanin did not change the methylation of GSTP1 in HepG2 cells either.~~ Authors believe that in human non-tumorous liver cells, betanin may induce the expression of phase II detoxifying enzymes through Nrf2 activation as a result of the mitogen-activated protein (MAP) kinases stimulation. Furthermore, in their previous *in vivo* study on rats (Krajka-Kuźniak et al. 2012), they found that betanin is responsible for the hepatoprotective activity of RBR, and through induction of detoxifying/antioxidant enzyme expression may play an important role in the prevention of liver injury and cancer. (Krajka-Kuźniak et al. 2013). In Acute Myocardial Infarction (AMI) model of rat, treatment with betanin reduced oxidative damage by reversing isoproterenol-induced effects such as inhibition of superoxide dismutase (SOD), catalase (CAT), GSH activities and increasing of malondialdehyde (MDA) levels and MPO activity (Yang et al. 2016). Betanin dose-dependently attenuated the xenobiotic-induced oxidative stress in rats kidney (Tan et al. 2015), liver

(by reversing paraquat-induced elevated levels of cytochrome P450 3A2 mRNA expression) (Han et al. 2014) and lung (by reversing paraquat-induced elevated levels of MDA, and MPO activity, and reduction of SOD activity) (Han et al. 2015a). Co-treatment of Caco-2 cells with IL-1β and 5–25 μM indicaxanthin (nutritionally relevant dose), dose-dependently prevented ROS formation and loss of thiols in these cells (Tesoriere et al. 2014). However, another study revealed that treatment of LPS-stimulated murine macrophage cell with indicaxanthin between 0.5–3 hours, moderately inhibits ROS production, and dose-dependently elevates conjugated diene (CD) lipid hydroperoxides as well as the production of hydroxynonenal-protein adducts. It also showed that at the time of 3–12 hour after exposure, indicaxanthin dose-dependently and progressively increases ROS production. It appears that LPS induces pro-oxidant activity of indicaxanthin (Allegra et al. 2014a). Oxysterol-induced RBCs eryptosis was prevented by concurrent exposure to 5 μM-indicaxanthin ((The mixture of oxysterols at final concentrations of 7mM-7-KC, 2mM-TRIOL, 4mM-a-epox, 1mM-7a-OH, 2mM-7b-OH and 4mM-b-epox (20 mM of total oxysterols) was added to the cells using tetrahydrofuran at a 0·1% (v/v) final concentration)), through reduction of ROS production and elevation of GSH levels. Incubated RBCs with oxysterol mixture (see above) plus 5 μM-indicaxanthin lost the ability of adherence to HUVECs (human umbilical vein endothelial cells) monolayers. It is suggested that indicaxanthin treatment can prevent vascular tissue injury and consequent thrombotic complications in hypercholesterolemia (Tesoriere et al. 2015). RBR feeding in rats, had a positive effects on redox state and increased reducing power, H-donating ability, free SH group (thiol) levels of liver and plasma as well as liver concentrations of the glutathione peroxidase (GPx) ,SOD, zinc and copper content which are necessary for the functioning of these antioxidant enzymes (Váli et al. 2007). Microsomal lipid peroxidation (expressed as TBARS concentration) in the rat's liver increased via administration of N-nitrosodimethylamine (NDEA) or carbon tetrachloride (CCL_4), but pretreatment with RBJ before the CCl4 and NDEA administration resulted in a 38%

reduction and a further elevation in TBARS, respectively. Both xenobiotics inhibited the activity of antioxidant enzymes by 26-77%. However, consumption of RBJ partially recovered the activity of GPx, Glutathione reductase (GR), and SOD activity, by 35%, 66%, and about 3-fold, respectively. RBJ consumption in NDEA exposed rats could lower the xenobiotic mediated elevation of plasma protein carbonyls up to 30%. DNA damage levels in blood leukocytes were reduced by 20% via pretreatment with RBJ, in comparison with NDEA alone and were increased by 40% for CCl4 treated group (Kujawska et al. 2009). Feeding of rats with RBJ may decrease the activities of cytochromes P450, CYP1A1/1A2, and CYP2E1. NDEA treatment reduced the activities of these enzymes with spontaneous increase in the activity of CYP2B. Moreover, combination of RBJ and NDEA treatments, significantly enhanced CYP2B activities. Modulation of P450 enzyme activity was accompanied by changes in the relevant substrate proteins levels. Cytosolic NQO-1 (phase II enzyme) showed the most significant elevation in level and activity. RBJ may protect against N-nitrosodiethylamine (NDEA)-induced liver injury via reduction of DNA damages (Krajka-Kuźniak et al. 2012). Moreover, in another experimental study on rats, the activities of cytochrome CYP1A1/1A2 was decreased and phase II enzymes were increased via RBJ feeding, and treatment with 7, 12-dimethylbenz[a]anthracene (DMBA), alone or in combination with RBJ, increased the activities of all Phase I and II enzymes and cytosolic NAD(P)H:Quinone. Similar to previous study, among the increased enzymes, the elevation in activity and levels of Oxidoreductase-1 was the highest. In the mammary gland, the levels of glutathione s-transferase pi (GSTP) significantly increased through treatment with RBJ (20% elevation), DMBA alone or in combination with RBJ. Briefly, RBJ showed tissue-specific effects dependent on the carcinogen's type (Szaefer et al. 2014).

In an in-vitro study, the reducing power and free radical scavenging activity of five RBR pomace extracts (Detroit, Cardeal-F1, Egyptian, Bicor, and Kestrel) were determined spectrophotometrically. The reducing power of these five extracts were dose-

dependent and among these, Detroit RBR pomace extract containing 37.22 ± 1.62 mg betanin/100 g $_{d.w.}$ had the most antiradical activities (high reducing power; EC_{50} = 123.39 ± 06.05 µg/ml and 83% inhibition of DPPH radicals; EC_{50} = 2.06 ± 0.10 µg/ml). Also, in treated rats with Detroit RBR pomace extract and then CCl_4, 2, 3 ml Detroit/kg $_{b.w.}$ had the best neutralizing effect against CCl_4-induced oxidative stress (significantly decreased glutathione peroxidase level of liver and increased catalase and glutathione levels of liver, near to the physiological levels) and hepatoprotective effect (through inhibition of the adverse effects of CCl_4-induced free radicals). It appears that phenolics and betalains have affected antioxidant and hepatoprotective status (Vulić. 2014). In hypertensive patients, supplementation with RBJ, not CRB, increased total antioxidant capacity percent of their RBCs (pre-supplementation=74.79±2.53, post-supplementation=77.86±2.71; p=0.001), thus reduced free radical-induced erythrocyte hemolysis and the concentration of released hemoglobin (Asgary et al. 2016). RBR products, in both the liquid and solid state, had antioxidant effects, and their betalains dose-dependently inhibited oxidative metabolism in isolated neutrophils from obese participants (ROS production was significantly higher in the neutrophils of obese individuals compared to healthy individuals) (Zielińska-Przyjemska et al. 2009). In a human study, consumption of betalain-rich RBR extract (dosages > 35 mg) beneficially affects Osteoarthritis pains, probably by inhibiting active neutrophils hypochlorous acid-induced protein oxidation (serum levels of advanced oxidation protein products was reduced by up to 48%) (Pietrzkowski et al. 2010). Supplementation of gentamicin-stimulated rats with RBR ethanol extract restored the renal endogenous antioxidant status (El Gamal et al. 2014). In rats, administration of RBR crisps increased GPx activity compared to treatment with dyslipidemic diet (Wroblewska, Juskiewicz, and Wiczkowski. 2011).

Total antioxidant capacity in nine species of PPFJ (*Opuntia robusta Gavia*, *Opuntia streptacantha Cardon*, *Opuntia ficus indica (OFI) Pelón*, *Opuntia Violaceae Moradillo, Opuntia leucotricha Duraznillo Blanco, Opuntia leucotricha Duraznillo Rojo, Opuntia robusta*

Taponc, *Opuntia robusta Amarillo* and *Opuntia rastrera Rastrero*) was 17-25 µmoles Trolox eq./mL, and they had inter-varietal differences in properties, phytochemicals content, and prevention of oxidative stress and cancer. Among MCF-7, PC3, Caco2 and HepG2 cancer cell lines, PPFs have negative effects mostly on the prostate and colon cell viabilities. Among that nine species of PPFs, *Moradillo* (with the highest flavonoid content) had the highest impact on the cell viability (Flavonoids contents of PPFJ= 95-374 µg quercetin eq/g). *Rastrero* reduced the growth of that four cancer cell lines (MCF-7, PC3, Caco2 and HepG2) without affecting normal fibroblast viability (Chavez-Santoscoy, Gutierrez-Uribe, and Serna-Saldívar. 2009). In healthy people, consumption of *OFI* fruit pulp had positive effects on the body's redox balance in parallel with decreased lipid oxidation possibly due to the betalain content of PPFs (Tesoriere et al. 2004b). In patients with familial hypercholesterolemia, regular consumption of *Opuntia robusta* for 4 weeks, significantly reduced oxidation injury (Budinsky et al. 2001). In healthy humans, isolated RBCs at 3 and 5 hours after PPF supplementation, showed a significant delay (33.0±4.5 min and 16.0±2.0 min, respectively) against an *ex vivo* induced hemolysis in comparison with the RBCs that were isolated before the supplementation. Neither vitamins C and E nor GSH was modified in the RBCs at any time point. Also, isolated RBCs after a 12-h fasting which were incubated with the purified betalains and then exposed to cumOOH, exhibited a more resistance to the hemolysis by the cumOOH, which was positively correlated (r^2=0.99) to the amount of the incorporated betalains. betanin and indicaxanthin demonestrated dose-dependent (at µM levels) comparable effect on the resistance to the *ex vivo* induced hemolysis. (Tesoriere et al. 2005).

Radical scavenging activity of Betalains:

Radical scavenging activity of betalains is comparable with the widely used synthetic antioxidant butylated hydroxytoluene (Pavlov et al. 2005). Betalain incorporation by isolated LDLs from human pooled plasma made them more resistant to copper-induced oxidation. The incorporated indicaxanthin appeared twice as effective as betanin in elongation of the time preceding the onset of oxidation (lag phase). During the inhibition period of lipid oxidation, betanin and indicaxanthin were consumed and delayed LDL-beta carotene consumption. Indicaxanthin, not betanin prevented vitamin E consumption at the beginning of LDL oxidation and prolonged its utilization time. Interaction with vitamin E, remarkably potentiate indicaxanthin for LDL protection. Betanin or indicaxanthin over a large concentration range had no pro-oxidant properties when added to the LDL system undergoing a copper-induced oxidation (Tesoriere et al. 2003). In plasma LDL isolated 3 h after consumption of PPF meal by healthy humans, elongation of the time preceding the onset of oxidation (lag phase) was longer than in the LDL isolated at 5 h, and no significant increment of lag phase was observed in LDL isolated at 8 h. Resistance to *ex vivo*–induced oxidative injury increased via elevation of incorporated betalains (Tesoriere et al. 2004a).

Allegra, Mario et al. (2007), stated that consumption rate of 5µM betanin during the catalytic cycle of myeloperoxidase (MPO) was; in the presence of MPO/nitrite-dependent LDL oxidation (v_i =1.8 ×10^{-8} Ms^{-1}) > in the presence of nitrite (v_i =1.01 ×10^{-8} Ms^{-1}) > in the absence of nitrite (v_i =0.75 ×10^{-8} Ms^{-1}); thus, their study suggested that betanin reacts with $NO_2^{\cdot}$ and can scavenge as a lipoperoxyl radical-scavenger. In the presence of nitrate, betanin (0.5–5.0 µM) inhibited LDL oxidation by MPO/Glucose oxidase. (GOD)/glucose/nitrite, dose-dependently decreased propagation rate, without affecting the lag period (after 20 min), and at the highest concentration (10 µM), prevented CD formation over a period of 100 min. However, in the absence of nitrate, through LDL lipid oxidation by MPO/GOD/glucose, the formation of CD lipid hydroperoxide dose-dependently was delayed by betanin (0.5–10 µM),

and the length of lag phase linearly increased while increasing the betanin concentration. Betanin had no effects on the propagation rate and was consumed within the lag phase. NO_2 - produced by the catalytic activity of MPO on nitrite - resulted in LDL oxidation which is first counteracted by the endogenous LDL antioxidant (vitamin E) consumption; so for 20 min, betanin did not modify temporal disappearance of vitamin E. After that, betanin inhibited LDL oxidation by MPO and also by NO_2 through blunting of undesirable MPO-mediated actions of nitrite and modulation of NO_2 metabolism in a favorable direction. Although in the absence of nitrate, betanin initially delays the oxidation of lipids, after the betanin depletion by residual oxidized-MPO, it resumes. But in the presence of nitrate betanin reduced NO_2 to the NO_2·, it prevents the oxidation of LDL by NO_2 and oxidized MPO. It also restores NO_2· with oxidized-MPO, thereby reducing or preventing oxidation by betanin in a dose-dependent manner. The oxidation kinetics showed that oxidized betanin is as effective as intact betanin in inhibition of LDL oxidation. Kinetic measurements suggested that the antioxidant effect is possibly the result of various actions (Allegra, Tesoriere, and Livrea. 2007).

In Tesoriere et al. (2008) study, among the foods submitted to the simulated digestive process, post-intestinal digesta from raw RBR had the most potent inhibitory effect on Methyl Linoleate peroxidation (IC_{50}=30±2 µg of fresh weight). The inhibitory effect of pure betanin, indicaxanthin, and vulgaxanthin was very high and comparable with that of vitamin E (IC50= 1±0.1, 0.7±0.06 and 0.75±0.07 µM, respectively). However, authors presumed that dose-dependent inhibition of the methyl linoleate oxidation by post-intestinal digesta was not related to betalains content individually, but it is probably correlated with interactions between betalains and food matrix (Tesoriere et al. 2008).

Toxicity of Betalains:

Betanin (red beet powder) is an FDA approved food additive. In a study on rats, investigators showed that 35 weeks consumption of betanin (E162 dye) did not overt any toxicity (Lechner et al. 2010). Also, indicaxanthin and betanin were nontoxic for HUVEC within the concentration range of 1 to 10 µM and 1 to 50 µM, respectively (Gentile et al. 2004).

Betalains against toxicity:

Betanin has protective features against paraquat-induced toxicity in the rat liver (reversed histological changes, elevated levels of serum ALT and AST, mitochondrial damage indicated by mitochondrial membrane swelling and reduced mitochondrial cytochrome C, and apoptosis-inducing factor protein levels) (Han et al. 2014) , kidney (reversed histological damages, elevated serum and urine markers of kidney injury)(Tan et al. 2015) and lung (reversed histological changes) (Han et al. 2015a).

In mice irradiated by (Cobalt 60) ^{60}Co, γ irradiation significantly reduced the number of the white blood cells (WBC), the spleen and thymus index as well as the liver, spleen, and kidney activities of SOD, CAT, and GPx enzymes. Furthermore, significantly enhanced lipid oxidation in the liver, spleen, and kidney caused 20-fold elevation in the micronucleus rate of polychromatic erythrocytes compared to the control group. Administration of RBRs reversed these effects in a dose-dependent manner. The micronucleus rate of polychromatic erythrocytes in the bone marrow of mice treated with 5, 20 or 80 mg betalains/kg $_{b.w}$ was reduced to 18.67, 14.56 and 9.89%, respectively, compared to 30.78% in the group treated with 0 mg/kg $_{b.w}$ betalains. Therefore, these phytochemicals have radioprotective effects and favorably modulate the immune system (Lu, Wang, and Zhang. 2009).

Treatment of rats with methanolic extract of RBR counteracted CCl_4 induced hepatotoxicity and restored the liver enzyme activities of the alanine aminotransferase (ALT), aspartate aminotransferase (AST), Bilirubin, WBC, RBC, polycythemia vera (PCV) and hepatic

lobule architecture, near to normal level (Rose, Sudha, and Sudhakar. 2014). Also, treatment with RBR ethanol extract improved gentamicin-induced renal dysfunction and structural damage partly through reduction of oxidative stress in the rat kidney (El Gamal et al. 2014). Addition of 3% RBR crisps to the hyperlipidemic diet of rats, decreased serum activity of AST (Wroblewska, Juskiewicz, and Wiczkowski. 2011). Furthermore, rattreatment with *OFIJ*, significantly reduced CCl_4-induced elevated levels of serum ALT and AST, injuries and dysfunctional changes in the liver hepatocytes. Hepatic parenchyma was normal at 48 h, and the injury was fully restored at 72 h (Galati et al. 2005).

Anti-inflammatory effects:

Co-treatment of Caco-2 cells with 5–25 μM indicaxanthin (nutritionally relevant dose) and interleukin (IL)-1β, dose-dependently reversed the IL-1β mediated induction pro-inflammatory cytokines (IL-6 and IL-8, PGE_2), ·NO release and elevation of epithelial permeability. Furthermore, indicaxanthin inhibited nuclear factor-κB (NF-kB) activation and reduced the expression of Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) as well as the activation of NADPH oxidase 1 (NOX-1). Apparently, inflamed state of Caco-2 cells did not have any effect on the uptake of indicaxanthin (Tesoriere et al. 2014).

In a rat model of acute inflammation, oral administration of indicaxanthin before xenobiotic injection, reduced the exudate volume (up to 70%) and the number of leukocytes recruited in the pleural cavity (up to 95%) at 24 h in time- and dose-dependent manner. In lipopolysaccharide-induced inflamed macrophages of rats, pretreatment with indicaxanthin (2 μmol/$kg_{b.w}$) inhibited the carrageenan-induced release of PGE_2 (91.4%), IL-1b (53.6%), TNF-a (71.1%), and decreased the mRNA levels of IL-1b (34.5%), TNF-a (81.6%), COX2 (87.7%), iNOS (75.2%), as well as COX-2 (65.9%) and iNOS (71.9%) protein levels. This intervention also inhibited the

xenobiotic-induced NO release (67.7%) of the recruited leukocytes. The activation of NF-kB was time- and dose-dependently inhibited by indicaxanthin as well (Allegra et al. 2014b).

In the LPS-stimulated RAW 264.7 cells, the activity of indicaxanthin in the membrane led to the formation of signaling mediators which are able to modulate prostaglandin (PG) biosynthetic pathway. However, in the presence ofeither vitamin E or an inhibitor of NADPH oxidase, indicaxanthin had no effect on the PG metabolism in these cells. Also, indicaxanthin did not have any effect in the absence of LPS. Because at the membrane level, LPS induced pro-oxidant activity of indicaxanthin (Allegra et al. 2014a).

In AMI rats, betanin consumption inhibited NF-κB and iNOS protein levels in the heart (Yang et al. 2016). Subcutaneous administration of E162 dye reduced the inflammation rates of both precancerous esophageal lesions and papillomas in N-nitroso methyl benzylamine (NMBA)-stimulated rats (Lechner et al. 2010). Intraperitoneal administration of betalain (30–300 mg/kg $_{b.w}$) to rats, diminished carrageenan-induced paw edema and neutrophil migration to the paw skin tissue. Also, intraperitoneal post-treatment with betalain (100 mg/kg $_{b.w}$) significantly inhibited the induction of paw edema by carrageenan and complete Freund's adjuvant (10 µl/paw). In addition, subcutaneous/oraly treatment with betalain (100 mg/kg $_{b.w}$) inhibited the edema and reduced the carrageenan (500 µg/cavity)-induced recruitment of total leukocytes (mononuclear cells and neutrophils). It also increased vascular permeability in the peritoneal cavity (Martinez et al. 2015). In rats, betanin dose-dependently attenuated the paraquat-induced acute injuries partly via anti-inflammatory mechanisms in different tissues. In the lung, it can reverse the xenobiotic-induced elevated levels of lung/body weight, permeability, neutrophils infiltration, NF-κB, broncho-alveolar lavage fluid (BALF) IL-1 and TNF-α, and it reduces the levels

of claudin-4 and zonula occluden-1 protein levels and BALF IL-10 too (Han et al. 2015a). In the kidney, betanin can reverse xenobiotic-induced elevated levels of iNOS and COX, and it can activate NF-κB and lysosomal protease (Tan et al. 2015).

An in vitro study compared the semi-synthetic analogs of betalains and natural betalains potentials on inactivation of lipoxygenase and COX. These enzymes are involved in the inflammatory response. This study demonstrated that Phenethyl-amine-betaxanthin is the most potent inactivator of cyclooxygenase. Whereas the natural pigment betanidin and a betalain analog derived from indoline are the most potent inactivators of lipoxygenase (Vidal et al. 2014). Furthermore, RBR treatment reversed the pro-inflammatory effects of mitogens in peripheral blood mononuclear cells (PBMCs) (Winkler et al. 2005). Betalains and RBR products (in both the liquid and solid form) also showed anti-inflammatory effects in PMN cells (Zielińska-Przyjemska et al. 2009). In gentamicin-stimulated rats, ethanolic extract of RBRcaused a significant reduction of TNF-α, NF-kB, NF-kB-DNA binding activity, IL-6, and MPO activity in kidney after gentamicin-induced nephrotoxicity. Also, elevated Inflammatory infiltration caused by gentamicin was reduced in renal tubes, thus kidney damages were improved partly due to the reduction of inflammation (El Gamal et al. 2014). Insubjects with high levels of serum TNF-α (higher than 1 pg/mL at the beginning of the study) , supplementation with betalain-rich RBR extract reduced serum levels of TNF-α after 10 days (Pietrzkowski et al. 2010). In another human study, supplementation with RBJ or CRB significantly decreased Intercellular Adhesion Molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), high sensitive C reactive protein (hs-CRP), IL-6, E-selectin, and TNF-α. Reduction of hs-CRP and TNF-α in RBJ group was more than in CRB group, thus RBJ was more effective in improving systemic inflammation (Asgary et al. 2016). Betanin (5 μM) and indicaxanthin protect endothelium cells (HUVEC cells) against cytokine-induced redox state alteration through inhibition of ICAM-1 expression (30 and 17% inhibition by betanin and indicaxanthin, respectively) (Gentile et al. 2004). In a human study, the serum hs-CRP reduced

significantly after supplementation with PPFJ, not with apricot juice, and uric acid levels remained without any changes at healthy range (Jensen. 2016). In pre-diabetic humans, supplementation with OpunDia^{TM} (a preferred blend of *OFI* cladode and fruit skin extract) had no effects on hs-CRP (Godard et al. 2010).

Effects on blood pressure:

In a human study, 2 weeks' supplementation with RBJ and CRB significantly decreased both systolic and diastolic blood pressure. The effects of RBJ supplementation on outcomes were more effective than CRB (Asgary et al. 2016). Also, in another human study, after 3 weeks consumption of concentrated RBJ, systolic blood pressure was reduced significantly (−7.3 ± 5.9 mmHg); but, this effect was not maintained after 1-week post supplementation phase (2.8 ± 6.1 mm Hg) (Jajja et al. 2014). In addition, consumption of RBJ by humans accompanied with unrestricted diet, caused a trend to lower SBP by 4–5 mmHg at 6 h in men (Coles and Clifton. 2012).

Effects related to Glucose:

Betanin showed anti-fibrotic effect on rat heart; because it antagonized the effects of fructose treatment, oxidative stress, activation of fructose feeding-induced NF-κB, and protein glycation (via reduction of protein glycation reactive intermediate, advanced glycation end product and receptors for advanced glycation end products). Also, in bovine serum albumin/fructose system, betanin had an antiglycative effect, which supported that anti-glycation is one of the protective roles of betanin *in vivo*. Authors believe that betanin may be introduced as an adjuvant therapy for diabetic cardiomyopathy (Han et al. 2015b). Consumption of OpunDia^{TM} (a preferred blend of *OFI* cladode and fruit skin extract) in prediabetes obese subjects, significantly decreased blood glucose concentrations at the 60, 90 and 120 min times point (from 205.92 ± 36.90 to 188.84 ± 38.43, from 184.55 ± 33.67 to 169.74 ± 35.16, and from 159.24 ±

17.85 to 148.89 ± 24.86 mg/dl, respectively) with the pre-oral glucose tolerance test (OGTT) compared to the OpunDia^{TM} bolus trial. Supplementation with OpunDia^{TM} had no effects on blood chemistry safety parameters, OGTT time points, area under the curve, insulin, proinsulin, HbA_{1c}, adiponectin, carbohydrates, fat, protein and total kcal, fat mass, fat free mass, percent body fat and total body weight during this study (Godard et al. 2010). Also, in healthy men who consumed *OFI* cladode and fruit skin extract, plasma insulin was increased at rest and after endurance exercise, and the clearance of an oral glucose load from the circulation was facilitated (Van Proeyen et al. 2012). The sugar content of PPFJs in Chavez-Santoscoy et al. 2009 study, was 8-14.7°Brix (Chavez-Santoscoy, Gutierrez-Uribe, and Serna-Saldívar. 2009). Also, consumption of RBJ beverage, a rich source of betalain degradation compounds (5975±68 mg/l) including the orange/yellow pigment neobetanin (5617±65 m/l), Isobetanin (131±15 mg/l), and 17-Decarboxy-Isobetanin (22.56±0.4 mg/l) significantly lowered glucose response in the 0-30 min phase compared to control beverage (Wootton-Beard et al. 2014). And in obese, not in non-obese adults, insulin sensitivity was improved following RBJ and glucose co-ingestion without inhibited nitrate reduction of oral bacteria, (Beals et al. 2017). Also, in rats, administration of RBR crisps resulted in a tendency towards a lower level of serum glucose (Wroblewska, Juskiewicz, and Wiczkowski. 2011).

Effects on Lipids:

In a human study, supplementation with RBJ, not CRB, decreased non-high-density lipoprotein-cholesterol (non-HDL-c), low-density lipoprotein-cholesterol (LDL-c) and total cholesterol (TC) (Asgary et al. 2016). In AMI rat, treatment with betanin reduced low-density lipoprotein (LDL) level (Yang et al. 2016). Consumption of a dyslipidemic diet in rats increased their serum TC, TC/HDL-c ratio, atherogenic index, and triacylglycerols (TAGs), and also suppressed the production of short-chain fatty acids (SHFAs).

However, administration of RBR crisps prevented the elevation of TC and TAG and resulted in a tendency towards higher total SCFAs pool. Administration of 3% RBR crisps decreased hepatic TC level. Therefore, the results of this study suggested that the addition of RBR crisps could alleviate metabolic changes in dyslipidemic diet-administered rats (Wroblewska, Juskiewicz, and Wiczkowski. 2011). But in another rat study, RBR feeding decreased the concentration of SCFAs, however, increased the concentration of long-chain fatty acids (Budinsky et al. 2001; Váli et al. 2007).

Effects on the Cardiovascular system:

According to previously described studies, betalains show several favorable effects on cardiovascular risk factors such as lipid profile, lipids oxidation, and inflammation.

In a study on AMI model of rats, cardiac function and infarct size were improved, and AMI was ameliorated by administration of betalains (Yang et al. 2016). In rats, intravenous (i.v.) injection of betanin affected the cardiovascular system and had favorable effects on the heart rate and blood pressure. In addition, the magnitude of the contractions of an isolated portal vein (in vitro) was increased transiently. The former effect of 0.9 µmol betanin was equivalent to about 2 nmol adrenalin (i.v. injection). Pretreatment with i.v. injection of propranolol (0.3 µmol) could not block the betanin effects on cardiovascular function. Specific adrenergic and cholinergic blockers were partially blocked by betanin *in vivo* and *in vitro* effects. The exact mechanism of betanin in cardiovascular function remains unclear up to now. Also, addition of 0.9 µmol betanin to the isolated perfused liver of rats led to a small decrease in the liver blood flow and a concomitant decrease in the bile flow, which lasted about 30 min and returned to the normal values after 1 h. These effects may have been due to a vasoconstricting effect of betanin (Krantz, Monier, and Wahlström. 1980). Furthermore, human

supplementation with RBJ and CRB significantly increased flow-mediated dilation (FMD). The elevation of FMD in CRB group was higher than in RBJ group, thus RBJ acts more effective than CRB in the endothelial function improvement (Asgary et al. 2016). In another human study, the postprandial impairment of FMD following a mixed meal was attenuated by the consumption of RBJ (Joris and Mensink. 2013). In Oliveira et al. study, FMD (77%), blood flow velocity (BFV=31%), and reactive hyperemia (RH=18%) were increased by consumption of RBR-based nutritional gel in humans (de Oliveira et al. 2016).

Effects on the Nervous system:

In a study on rats, administrated indicaxanthin, in an amount compatible with a dietary consumption of PPF in human, crossed the rat blood-brain barrier, and accumulated in the brain. It affected the bioelectric activity of hippocampal neurons locally treated with amounts comparable with those measured in the brain and modulated glutamate-induced neuronal excitation. Thus indicaxanthin may act at the glutamatergic synapses. In Silico molecular modeling showed that N-methyl-D-aspartate receptor (NMDAR) may be an indicaxanthin target (Allegra et al. 2015).

Effects on the Gastrointestinal system:

Extract of *OFI* yellow fruit pulp (*Sulfarina*) had direct antispasmodic effects on the intestinal motility and reduced the spontaneous contractions of rat's ileum in a dose-dependent manner. This effect was not influenced by Nω-nitro-L-arginine methyl ester (Nitric oxide synthase blocker), tetrodotoxin (neuronal blocker), atropine (muscarinic receptor antagonist) or tetraethylammonium (potassium channel blocker). The contractions were evoked by carbachol, not by high extracellular potassium. However, contractions were effectively inhibited by *OFI* fruit extract through intracellular Ca^{2+} release pathways in the smooth muscle cells. Authors believe that

indicaxanthin appears to be the principal constituent responsible for the *OFI* fruit extract induced effects (Baldassano et al. 2010). Also, indicaxanthin has remarkable spasmolytic effects on the intestinal contractility in mice and reduces the contractility of ileal longitudinal muscle by inhibiting phosphodiesterases and increasing cAMP concentration. Indicaxanthin increased both basal and forskolin (an adenylyl cyclase activator)-induced cAMP content of mouse ileal muscle. Therefore, indicaxanthin can be used in the treatment of motility disorders, such as abdominal cramps (Baldassano et al. 2011).

Apoptotic effects:

In a study, treatment of Caco-2 cells or neutrophils of patients with inflammatory bowel disease with 100 μM betanin, increased DNA damages. DNA damage in Caco-2 cells was less than in neutrophils. In Caco-2 cells, H_2O_2 treatment led to a 4-fold elevation in DNA strand breaks in comparison with control cells, and betanin pre-treatment decreased DNA damage in these cells. Authors presumed that betanin may act as a pro-apoptotic factor in inducing procaspase-3 cleavage, activating caspase-3 and arresting mitochondrial transmembrane potential (Zielińska-Przyjemska et al. 2016). Also, betanin treatment (2–500 μM) enhanced caspase-3 activity in phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils (PMA induces oxidative stress in neutrophils), but had no effects in unstimulated neutrophils. In addition, elevation in products of caspase-3 cleavage was detected in both unstimulated and stimulated neutrophils (Zielińska-Przyjemska et al. 2012). In another study, treatment with betanin/Isobetanin led to significant reduction in the cancer cells proliferation and viability, alteration of the mitochondrial membrane potential (particularly via both intrinsic and extrinsic apoptotic pathways), and formation of autophagosome vesicles in MCF-7-treated cells. Furthermore, it resulted in the autophagic cell death through high elevation of the apoptotic Bad, TRAILR4, FAS and p53 proteins expression. Authors conclude that

betanin/Isobetanin treatment may be useful in cancer therapy, especially in the functional p53 tumors. The betanin-enriched extract did not affect normal cell lines (Nowacki et al. 2015). Betanin decreased the proliferation of human chronic myeloid leukemia (K562) cell line (IC_{50}= 40 μM) in dose and time-dependent manner. In addition, treatment with 40 μM betanin, stimulated the cells to enter the sub G0/G1 phase (28.4% of cells), and it showed apoptotic properties such as chromatin condensation, cell shrinkage, membrane blebbing, DNA fragmentation pattern, poly ribose (ADP) polymerase cleavage, reduction in the membrane potentials, downregulation of Bcl-2 and the release of cytochrome c into the cytosol. Also, confocal microscopic data showed that betanin enters into the cells and induces apoptosis in K562 cells through the intrinsic pathway (Sreekanth et al. 2007). In rats, treatment with E162 dye increased the apoptotic rate compared to the controls. These may be due to the inhibition of oxygen radical-induced signal transduction by antioxidant effects of betalains or the effects of other constituents in E162 dye (Lechner et al. 2010).

Studies on human intestinal carcinoma cell lines showed that indicaxanthin has the potential to affect global DNA methylation, induction of cell growth arrest and reversal of oncosuppressor gene silencing (Livrea and Tesoriere. 2015). Indicaxanthin dose- and time-dependently decreased Caco-2 and Ha 22T cells proliferation (IC_{50}= 50 μM). Also, 100 μM of indicaxanthin at 48 and 72 h showed a pro-apoptotic effect and 10 μM of it induces a slight global demethylation (Naselli et al. 2012). Indicaxanthin decreased Caco-2 and Ha 22T cells proliferation (IC_{50}= 50 μM) in a dose- and time-dependent manner. Also, 100 μM of indicaxanthin at the times of 48 and 72 h showed a pro-apoptotic effect and 10 μM of it induced a slight global demethylation (Naselli et al. 2014). Also, indicaxanthin is a potential epigenetic agent for protecting colon cells against tumoral alterations. An in Silico molecular modeling approach suggested that indicaxanthin can stably bind to the catalytic site of DNMT1, and inhibits its activity, thus it increases the

expression of DNA methyl transferases (DNMT) gene as well as other genes involved in DNA demethylation (Naselli et al. 2015). Oxysterol-induced eryptosis which occurred through the followings: early production of ROS, releas of prostaglandin E_2 (PGE_2), PGE_2-dependent Ca channels opening, externalization of membrane phosphatidylserine and cell shrinkage. Tesoriere et al revealed that treatment with indicaxanthin (1–5μM) could inhibit the number of these items including GSH depletion, ROS production, Ca^{2+} entry and PGE_2 release. They concluded that indicaxanthin could prevent cell shrinkage and phosphatidylserine externalization in a dose-dependent manner (Tesoriere et al. 2015).

Also, in a study on the human monocyte/macrophage cell line, indicaxanthin counteracted all the 7-ketocholesterol induced pro-apoptotic effects, thiol depletion and the activation of NF-kB. 7-ketocholesterol is a component of oxidized LDL which plays an important role in the atherosclerosis pathogenesis. Authors presumed that these effects of indicaxanthin may be ascribed to the inhibition of NF-kB activation, inhibition of NOX-4 basal activity and over-expression, maintaining of cell redox balance and Ca homeostasis, prevention of mitochondrial damage and consequently apoptosis (Tesoriere et al. 2013a).

RBRJ at the concentrations of 0.1-10% exhibited pro-apoptotic effects on the stimulated neutrophils cell line at the time of 24 h (Zielińska-Przyjemska et al. 2009). In addition, treatment of gentamicin-stimulated rats with RBR ethanol extract improved their kidney damages, partly via decreasing apoptosis. Apoptosis occurred through the reduction of cleaved caspase 3, expression of Bax protein and elevation of the Bcl-2 protein expression (El Gamal et al. 2014). In a cell study, the cytotoxic effects of the RBR extract are compared with doxorubicin which is a red colored anticancer antibiotic, and remarkably similar to the betanin molecular structure. Both of them exhibited a dose-dependent cytotoxic effect in MCF-7 and PC-3 cancer cell lines. Although, the RBR cytotoxicity was

significantly lower than doxorubicin in cancer (PC-3), normal human skin (FC) and liver (HC) cell lines. Authors presumed that betanin may play an important role in the cytotoxicity exhibited by RBR (Kapadia et al. 2011).

Conclusion: Based on the current literature review, we found many studies generally about the biological effects of prickly pear fruit and red beetroot crude extracts. Due to the presence of various bioactive components such as vitamins, nitrate, betaine and polyphenols in crude extracts and their different activities, understanding of the particular biological functions of betalains individually in the betalain-rich sources of our diet seems to be difficult, and further *in vivo* and *in vitro* investigations on high-purity betalains (especially betanin and indicaxanthin) are necessary.

In summary, natural betalain pigments can serve not only as safe additives to add natural color or antioxidant properties to foods, pharmaceuticals, and cosmetic, but they also possess promising beneficial potential as therapeutic products to promote health and prevent diseases such as hypertension, dyslipidemia, cancer, and vascular stenosis, among others..

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Table 1: Human studies about Betalains/Betalains-rich food

Subjects	Design & Treatment	Methods	Ref.
24 hypertensive P, Age= 25–68, BMI= 24.73 ± 8.21	In an un-blinded controlled cross-over pilot RCT, P received 2 treatments (250 ml RBJ AND 250 g CRB; daily for 2 wks) on 2 occasions which separated by 2-wks washout.	-Flow-mediated dilation: GE Vivid 3 Ultrasound apparatus	(Asgary et al. 2016)
		-lipid profile, high sensitive C reactive protein and Fasting Blood Sugar levels: enzymatic assay on	

		an autoanalyzer	
		-Serum intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin and interleukin-6: ELISA kits	
		-non-high density lipoprotein-cholesterol: by subtracting high-density lipoprotein-cholesterol from total cholesterol levels	
		-Total antioxidant capacity: a cell-based erythrocyte hemolysis inhibition assay	
8 white (M) H in each part (3 M in both parts), Age= 19-40 [Age= 25.3 ± 1.3, BMI= 24.5 ± 1.7 (standard) & Age= 29.1 ± 1.3, BMI= 25.2 ± 2.0 (post-methionine conditions)]	In a two-part controlled cross-over RCT, P received 6 treatments (500 mg betaine supplement AND 517 mg high-betaine meal AND 500 mg choline supplement AND 564 mg high-choline meal AND 517 mg betaine + 622 mg choline AND a low betaine + choline meal as a control; single dose, under standard conditions or post-methionine load) on 6 occasions which separated by 6-days washout.	-Plasma and urine betaine, dimethylglycine, and carnitine: HPLC	(Atkinson et al. 2008)
		-Plasma homocysteine: fluorescence polarization on an Abbott IMX Analyzer	
		-Urine creatinine: using the Jaffé reaction on the Abbott Aeroset Analyzer	
		-Urine betaine and dimethylglycine excretion: as a ratio to Creatinine	
		-Serum Vitamin B-12 and red blood cells folate concentrations: separate	

		competitive immunoassays on an automated Chemiluminescence Analyzer	
		-Total choline in the food: Nuclear magnetic resonance spectroscopy	
12 (6 F, 6 M) non-obese P, Age= 25 ± 3, BMI= 26.3 ± 0.8 & 10 (8 F, 2 M) obese P, Age= 43± 4, BMI= 34± 0.8	In a controlled cross-over RCT, P received 2 treatments ((500 ml RBJ, supplemented with 25 g of glucose (carbohydrate load: 75 g), with AND without prior use of antibacterial mouthwash to inhibit nitrate reduction to nitrite; single dose) on 2 occasions which separated by 7-28-days washout. Actually, two modified oral glucose tolerance tests were administered, on two separate occasions.	-Glucose concentrations: automated device	(Beals et al. 2017)
		-insulin concentrations: ELISA	
		-Insulin sensitivity: the Matsuda Index, Mastuda, and DeFronzo, 1999 method	
15 (8 F, 7 M) P with sFHH, Age= 24-45 (33.1 ± 6.3), Height= 173.80 ± 3.97, Weight= 70.69 ± 3.22	In an interventional trial (design type: unclear; likely before & after or with non-randomized control), P received dietary counseling; weekly for 4 wks. Then, they received 250 g broiled edible pulp of *Opuntia robusta*; daily for 4 wks.	-lipids, lipoproteins, total cholesterol and triglyceride: Enzymatic methods	(Budinsky et al. 2001)
		-Oxidation injury via 8-epi-PGF2a in plasma, serum, and urine: ELISA	
10 (M) non-smoking H, Age= 23	In a placebo-controlled cross-	-Venous plasma samples were	(Clifford et al. 2016)

± 3, Height=1.82± 0.6, Weight=78.8± 6.7	over RCT, P received 3 treatments (250 ml RBJ AND 300 g CRB AND 250 ml of an isocaloric placebo drink; single dose) on 3 occasions which separated by 7-days washout.	collected pre, 1, 2, 3, 5 and 8h post-ingestion.	
		-betalain content: reverse-phase HPLC and mass spectrometry detection.	
		-Antioxidant capacity: Trolox equivalent antioxidant capacity (TEAC) assay	
		-Polyphenol content: Folin–Ciocalteu colorimetric methods (gallic acid equivalents)	
30 (15 F, 15 M) H, Age= 42.5 ± 3.4, BMI= 28.2 ± 1.3	In a double-blinded placebo-controlled cross-over RCT, P received 2 treatments (500 g juice containing 72% RBR+ 28% apples +15 mmol nitrate/L AND 500 g apple juice concentrate as a placebo; single dose) on 2 occasions which separated by 2-wks washout.	-blood pressure: an ambulatory blood pressure monitor	(Coles and Clifton. 2012)
20 (13 F, 7 M) P who were taking 1-3 anti-hypertensive medications, Age= 70.5 ± 5.6, BMI= 30.2 ± 5.3	In a double-blinded placebo-controlled cross-over RCT, P received 2 treatments (100 g RBR-based nutritional gel AND 100 g N-depleted gel as a placebo; single dose) on 2 occasions which separated by 1-wk washout.	-Arterial stiffness: via reductions in pulse wave velocity, augmentation index, stiffness parameter, and increases in the pressure-strain elasticity modulus, and arterial compliance by a method based on Doppler probe	(de Oliveira et al. 2016)
		-systolic/diastolic blood pressure and heart rate: Omron blood	

		pressure monitor	
		-Urinary nitrite and nitrate: HPLC	
		-Changes in the endothelial function: via increases in the flow-mediated dilation, reactive hyperemia, and blood flow velocity	
6 (F) non-smoking H, Age= 23-24, BMI= 19.1- 22.6	In a open-labeled controlled cross-over pilot RCT, P received 2 treatments (500 ml of a commercial RBJ, containing 362.7 mg betanin AND 500 ml tap water; single dose) on 2 occasions which separated by 1-wk washout.	-Urine was collected in 1-h and 2-h intervals up to 24 h post-supplementation	(Frank et al. 2005)
		-Renal elimination of betalains: spectrophotometer	
29 pre-diabetic obese P, Age= 20–50, BMI= 30–35	In a double-blinded placebo-controlled parallel RCT, 2 groups received either 200 mg OpunDia™ (blend of *OFI* cladode and fruit skin extract) OR microcrystalline cellulose as a placebo; daily for 16 wks. The acute phase consisted of an oral glucose tolerance test with 400 mg OpunDia™ given 30 min before orally ingesting a 75 g glucose drink.	-Plasma levels of glucose, insulin, high sensitive C reactive protein, adiponectin, proinsulin and glycosylated hemoglobin: ELISA	(Godard et al. 2010)
		-Body composition: air-displacement plethysmography (BOD POD)	
		-Macronutrient content: DINE Healthy Nutritional Software	
21 (9 F, 12 M) non-smoking, Age= 62.0 ± 1.4, BMI= 30.1 ± 1.2	In an un-blinded controlled parallel RCT, 2 groups	-blood pressure (resting clinic blood pressure, 24-hour ambulatory	(Jajja et al. 2014)

	received either 70 ml concentrated RBJ containing 300-400 mg nitrate/bottle, OR 200 ml blackcurrant juice containing 2.7 ± 0.1 mg nitrate/bottle as a control; daily for 3 wks. This study had a 1-wk post supplementation phase.	blood pressure monitoring, and home monitoring of daily resting blood pressure): Omron automated blood pressure monitor	
		-Urine and Saliva nitrate concentrations: gas chromatography-mass spectrometry	
196 P, Age: 33-82	In a double-blinded controlled parallel pilot RCT, 2 groups received either 177.5 ml Nopalea™ (PPFJ) OR apricot juice as a control; daily for 12 wks.	-Serum C-reactive protein and uric acid were measured at baseline and after 8 and 12 weeks.	(Jensen. 2016)
20 (M) overweight and slightly obese H, Age= 61 ± 7, BMI= 30.1 ± 1.9	In an open-labeled controlled cross-over RCT (or basic science), P received 2 treatments (a muffin containing 56.6 g fat + 140 ml concentrated RBJ AND a muffin containing 56.6 g fat + 140 ml drink as a control; single dose) on 2 occasions which separated by at least 1-wk washout.	-Flow-mediated dilation: Echo-Doppler	(Joris and Mensink. 2013)
		-Pulse Wave Analysis: Tonometer	
		-Plasma nitrite, nitroso/nitrosyl NO species: triiodide/ozone-based chemiluminescence	
24 P with Osteoarthritis	In a randomized open-labeled before & after clinical discovery, 3 groups received	-Standard serum biochemistry analysis was performed.	(Pietrzkowski et al. 2010)

	either 35 OR 70 OR 100 mg betalain-rich RBR (depleted of sugars and enriched in betalains up to 24%); twice daily for 10 days.		
		-McGill pain score system and Energy Score: Questionnaire	
		-Serum advanced oxidation protein products: kit, this assay measures serum proteins modified by chloramine or hypochlorous acid.	
		-Serum Cytokines and chemokines: Qynsys Inc. Array	
8 (5 F, 3 M) non-smoking H, Age = 32.65 ± 10.11, BMI = 21±2.0	In an interventional trial (design type: unclear; likely before & after study), P followed a betalain-free diet for 7 days and after an overnight fasting received a single dose of 500 g silician *OFI* fruit pulp, containing 28 mg indicaxanthin and 16 mg betanin.	-LDL preparation: isolated from EDTA plasma by ultracentrifugation, be free of other lipoproteins by electrophoresis on agarose gel, EDTA/salts removing by gel filtration on Sephadex G-25, and proteins determination by colorimetric method	(Tesoriere et al. 2004a)
		-LDL oxidation: incubation with oxygen-saturated EDTA-free phosphate-buffered saline, supplemented with CuCl2 as a pro-oxidant and spectrophotometrically monitoring of the conjugated diene lipid hydroperoxides formation.	
		-betalains measurement in plasma, urine, and LDL: HPLC	
		-Vitamin E and β-carotene measurement in LDL: HPLC	
18 (10 F, 8 M) H, Age= 33.33 ± 11.27, BMI= 23 ± 2.5	In a controlled cross-over RCT, P received 2 treatments (250 g *OFI* fruit pulp AND	-Total plasma F2-isoprostanes (8-epi-prostaglandin F2): gas chromatography-mass spectrometry	(Tesoriere et al. 2004b)

	75 mg vitamin C; twice daily for 2 wks) on 2 occasions which separated by 6-wks washout	with negative chemical ionization	
		-Plasma malondialdehyde: colorimetric reaction with thiobarbituric acid	
		-The ratio of reduced to oxidized glutathione in red blood cells (GSH: GSSG): Glutathione measurement by the 5,5'-di thio bis–reductase recycling method	
		-Plasma total antioxidant capacity: Trolox equivalent antioxidant capacity assay and HPLC	
		-LDL preparation: isolated from EDTA plasma by ultracentrifugation, be free of other lipoproteins by electrophoresis on agarose gel, EDTA/salts removing by gel filtration on Sephadex G-25, and proteins determination by colorimetric method	
6 (M) physically active H, Age= 21.0 ± 1.6, Weight= 78.1 ± 6.0	In a double-blinded placebo-controlled cross-over RCT, P received 2 treatments ((1000 mg *OFI* cladode and fruit-skin extract AND 1000 mg LUVOS Heilerde as a placebo; single dose) 30 min before and immediately after undergoing a 2-h oral glucose tolerance test at rest. After that, a 30-min cycling bout at ~75% VO2 max, and another 2-h oral glucose tolerance test after exercise were done) on 2	-Rate of maximal oxygen uptake ($VO2_{max}$) and the corresponding workload: using a maximal incremental exercise test on a bicycle ergometer (the workload that corresponded with ~75% of $VO2_{max}$ was calculated for each individual and used)	(Van Proeyen et al. 2012)

	occasions which separated by 2-wks washout.		
		-Oral glucose tolerance test $_{Rest}$: 75 g glucose was ingested, and blood samples were collected at $t_{0\ min}$, $t_{30\ min}$, $t_{60\ min}$, $t_{90\ min}$, and $t_{120\ min}$.	
		-Oral glucose tolerance test $_{Exercise}$: an extra 75-g oral glucose was administered at $t_{60\ min}$.	
16 (10 F, 6 M) H, Age: 27 ± 5, BMI= 23.3 ± 2.8	In a single-blinded placebo-controlled cross-over RCT, P received 3 treatments (225 ml RBJ beverage AND 225 ml glucose beverage AND 225 ml placebo beverage; single dose) on 3 occasions which separated by at least 2-days washout.	-Nitrate and nitrite concentrations: gas chromatography-mass spectrometry	(Wootton-Beard et al. 2014)
		-betalains and phenolic compounds: HPLC	
		-Index of insulin sensitivity (SI): Burattini et al, 2009 method	

CRB=cooked red beet, ELISA: Enzyme-linked immunosorbent assay, F=female, H= healthy participant, HPLC: high-performance liquid chromatography, M=male, *OFI=Opuntia ficus indica*, P=participants/patients, PPFJ= prickly pear fruit juice, RBJ=red beet juice, RBR=red beetroot, RCT= randomized clinical Trial, sFHH= severe familial heterozygous hypercholesterolemia, wk=week (Age=years, BMI=kg/m^2, Height=m, Weight=kg)

Table 2: Animal studies about Betalains/Betalains-rich food

Animals	Treatment	Methods	Ref.
Wistar Harlan rats (M), W= 175–200	Parallel G (10 A/G) received either saline alone (C) or 0.5 or 1 or 2 µmol indicaxanthin/kg by oral gavage 30 min before and every 8 h after injection of	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions	(Allegra et al. 2014b)

	carrageenin, up to 40 h. Rats were killed at 4, 24, and 48 h after carrageenan injection, and pleural exudates were collected and processed. In some experiments, rats (10 A/G) received 3 mg indomethacin (an anti-inflammatory drug)/kg, orally, 30 min before carrageenin injection. Also, 2 µmol indicaxanthin/kg administered to measuring its urinary excretion and plasma concentration.	containing the pigment to cryodesiccation, and purification by semi-preparative HPLC (Stintzing, Schieber, and Carle. 2002).	
		-Induction of pleurisy: rats were slightly anesthetized, and carrageenin solution was injected into the right pleural cavity. Rats were killed 4, 24, and 48 h later and the exudate was harvested.	
		-Cell viability: MTT assay	
		-Differential cell count: May-Grunwald staining.	
		-Protein concentration: Bradford 1976, method (Bio-Rad kit)	
		-Total RNA isolation: TRIzol Reagent	
		-mRNA Levels of interleukin-1b, tumor necrosis factor-a, inducible NO synthase, and cyclooxygenase-2: Reverse transcription-polymerase chain reaction (β-actin= internal control)	
		-Levels of cyclooxygenase-2, inducible NO synthase, and β-actin proteins: Western Blot analysis	

		-NF-κB (p65) DNA-binding activity (as NF-κB activation indicator): electrophoretic mobility-shift assay in nuclear extracts	
		-Protein levels of PGE2 in the pleural exudates: Enzyme immunoassay kit	
		-Protein levels of tumor necrosis factor-a and interleukin-1b: ELISA kit	
Adult Wistar rats (M), W= 220–280	2 randomized G (10 A/G) received either 2 µmol/kg indicaxanthin or saline alone (C), by oral gavage. After were sacrificed after 1, 2.5, 3, 4 and 5 h. Brain samples were collected and processed. Also, 9 A were anesthetized for pharmacokinetic studies.	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing et al., 2002 method	(Allegra et al. 2015)
		-Quantification of brain's indicaxanthin: extraction with chloroform-methanol, analyzed with Varian Microsorb HPLC column and quantified via Spectrophotometer at 482 nm.	
		-Activity of neurons: Electrophysiological recordings	
		-Preparation of protein and ligand: protein preparation wizard of Glide software 5.9 for protein and LigPrep software 2.6 for band.	
		-Docking: Glide XP high-performance docking procedure	
		-Binding free energy calculation: Prime/MM-GBSA software 3.2	
		-Computational mutagenesis:	

		finding the contribution of specific residues to the protein function by mutating the residues to alanine to recognize structural and energetic features of hotspots. The interacting residues with indicaxanthin were mutated to alanine.	
C57BL/10SnJ mice (M), Adult, W= 25±1.5	Mice were killed by cervical dislocation. The ileum was removed and its segments (20 mm in length) were suspended in Krebs solution in a 4 channel organ bath to investigate the effects of yellow *OFI* pulp extract, on the motility of ileum.	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing et al., 2002 method	(Baldassano et al. 2010)
	-For obtaining dose-dependent response curves, *OFI* pulp extract (10-320 mg fresh pulp equivalents/mL of organ bath) was tested cumulatively into the bath for 5 min.	-Analysis of Ascorbic Acid: reversed-phase HPLC, Spectrophotometer	
	-Evaluation of the effect of *OFI* pulp extract in the presence of tetraethylammonium (20mM), tetrodotoxin (1 μM), atropine (1 μM), or N_{ω}-nitro-L-arginine methyl ester (300μM) was performed by adding them to the organ bath 30 min before *OFI* pulp extract.		
	-Evaluation of the effect of a 5min contact of *OFI* pulp extract (80-320mg/mL of organ bath) on the evoked-		

	contractions by either exogenous carbachol (10 μM) or high extracellular K^+ (60 mM) was performed.		
	-Evaluation of the ileal spontaneous mechanical activity was performed in the presence of ascorbic acid (18-600 μM) or indicaxanthin (3-100 M).		
	-Evaluation of the carbachol-induced contraction was performed in the presence of indicaxanthin (25-50-100 μM) or both ascorbic acid (600 μM) and indicaxanthin (100 μM).		
C57BL/10SnJ mice (M), Adult, W= 25±2.1	Mice were killed by cervical dislocation. The ileum was removed and its segments (20 mm in length) were suspended in Krebs solution in a 4 channel organ bath to investigate the effects of indicaxanthin, on the motility of ileum. The effect of 3 to 100 μM indicaxanthin was evaluated to obtain concentration-response curves. The contact time for each concentration was 5 min. Also, The effect of indicaxanthin was evaluated at least 30 min after addition of 10 μM of indomethacin (an inhibitor of cycloxygenase), 10 μM 2′5′ dideoxyadenosine (an inhibitor	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing et al., 2002 method	(Baldassano et al. 2011)

	of adenylyl cyclase), 10 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (a selective inhibitor of nitric oxide-stimulated guanylyl cyclase), 10 μM 3-isobutyl-1-methylxanthine (a non-selective inhibitor of cyclic nucleotide phosphodiesterase), 10 μM zaprinast (a selective inhibitor of the cGMP phosphodiesterase 5) to perfusing solution.		
		-Recording the mechanical activity of the mouse ileum longitudinal muscle: Power Lab/400 system	
		-cAMP content of ileal muscle: ELISA	
Wistar albino rats (F & M), Age= 8–10, W=180– 200	4 G (6 A/G) received either no treatment (C) or 85 mg gentamicin/kg (2 G of them, were orally pre-supplemented with 250 or 500 mg RBR ethanol extract/kg, i.p., for 20 days), for 8 days. Then blood samples were collected after 24 h.	-Scavenging Ability: DPPH assay	(El Gamal et al. 2014)
		-Acute Toxicity Test on rat: using oral administration of 50-2000 mg/kg of beetroot extract	
		-Serum Creatinine, uric acid, urea: Reflotron Plus Analyzer and Kits	
		-Kidney malondialdehyde: Utley et al., 1967 method	

		-Kidney catalase and total protein content: kit	
		-Kidney Nonprotein Sulfydryl's Content: Sedlak and Lindsay, 1968 method	
		-Kidney myeloperoxidase assay neutrophil recruitment: by means of myeloperoxidase activity: Krueger et.al. 1990 method	
		-Kidney levels of TNF-α and IL-6 proteins: ELISA kits	
		-Assessment of nitric oxide: Griess reaction	
		-Nuclear total protein extracts: Extraction kit	
		-Protein concentrations: Lowry et al., 1951 method	
		-Protein levels of NF-kB, β-actin, Caspase-3, Bax and Bcl-2: Western Blot analysis	
		-NF-kB (p65) Activation: ELISA	
Wistar rats (M), W= 180–200	4 randomized G (18 A/G) received either 3 ml water (C) orally, or 1 ml carbon tetrachloride (in olive oil 50%)/kg, i.p. 2 h later, 3 G of CCl_4-treated rats received either no further treatment or 3 ml *OFI* juice/rat, orally or 0.1 g silymarin/kg as a reference drug (dissolved in CMC 1%) orally. Also, another G (18 A) received 3 ml *OFI* juice/rat, orally, for 9 days. And 2 h after the last administration, treated with 1 ml CCl_4 (in olive oil 50%)/kg, i.p. Finally, 6 rats/G	-Apoptosis evaluation: terminal deoxynucleotidyl transferase dUTP nick end-labeling [TUNEL], Low et al., 1995 method	(Galati et al. 2005)

	at a time were killed after 24, 48 and 72 h, and their livers and blood samples were collected.		
		-All samples were observed and photographed: BH2 Olympus microscopy.	
SPF grade S-D rats (M), W=220 ± 20	4 randomized G (10 A/G) received either 25 (G III) or 100 (G IV) mg betanin/kg or the same volume of distilled water (C: I, II) by intragastric gavage, daily for 5 days. At the 3th day following administration of betanin, rats received 20 mg/kg of paraquat (II, III, IV) or normal saline (vehicle; I), i.p. A were sacrificed 24 hours after the last betanin administration and blood and liver samples were immediately collected.	-Liver injury: histopathology assay	(Han et al. 2014)
		-Serum levels of alanine aminotransferase and aspartate aminotransferase: kits	
		-Oxidative stress assay: (malondialdehyde assay: Saleem et al., 2000 method), (superoxide dismutase activity: Beauchamp and Fridovich, 1971 method), (catalase activity: Claiborne, 1985 method) and (glutathione: Zhang et al., 2003 method).	
		-Protein concentration: Bradford, 1976 method	
		-mRNA levels of P450 (CYP) 3A2 and β-actin: reverse-	

		transcription polymerase chain reaction	
		-Mitochondrial permeability transition assay: spectrophotometrically	
		-Liver mitochondrial protein extraction: kit	
		-Protein levels of cytochrome C, apoptosis-inducing factor, and β-actin: Western Blot analysis	
SPF grade S-D rats (M), W=220 ± 20	4 randomized G (10 A/G) received either 25 (G III) or 100 (G IV) mg betanin/kg or the same volume of distilled water (C: I, II) by intragastric gavage, daily for 5 days. At the 3rd day following administration of betanin, rats received 20 mg/kg of paraquat (II, III, IV) or normal saline (vehicle; I), i.p. A were sacrificed 24 hours after the last betanin administration and blood and lung samples were immediately collected.	-Total bronchoalveolar lavage fluid (BALF) cell count: hemocytometer	(Han et al. 2015a)
		-The number of PMNs in BALF: was calculated by multiplying the ratio of PMNs by the total cell count.	
		-Pulmonary permeability index: the ratio of protein concentration in BALF to that in plasma.	
		-Cytokine (TNF-α, IL-1b, and IL-10) concentrations in the BALF supernatants: ELISA kits	
		-lung injury: histopathology assay	
		-Oxidative stress assay:	

		(myeloperoxidase activity: Goldblum and Jay, 1985 method), (superoxide dismutase activity: Beauchamp and Fridovich, 1971 method), and (malondialdehyde level: Saleem et al., 2000 method)	
		-Protein levels of zonula occluden- 1, claudin-4, or β-actin: Western Blot analysis	
		-NF-κB (p65) DNA-binding activity (as NF-κB activation indicator): ELISA kit. Nuclear Protein was extracted from lung tissue.	
		-Protein concentration: Bradford, 1976 method	
Healthy S-D rats (M), W= 220 ± 20	4 randomized G (10 A/G) received either tap water (C) or 30% fructose tap water solution to induced heart fibrosis (G: II, III, IV) or 25 (G III) or 100 (G IV) mg betanin/kg, by intragastric gavage, for 60 days. After an overnight fasting, rats were sacrificed. Their blood and left ventricle were collected immediately to avoid the blood glucose fluctuation.	-Glucose concentration: Sasaki and Matsui, 1972 method	(Han et al. 2015b)
		-Glycated hemoglobin: Rao and Pattabiraman, 1990	
		-Insulin assay: ELISA kit	
		-Homeostasis model assessment: formula [insulin (μU/ml) × glucose (mmol/l)/22.5]	
		-Left ventricle injury: histopathology assay	

		-Immunohistochemistry staining: kit	
		-Protein levels of TGF-β1 and CTGF in left ventricle: Western blot analysis	
		-Chemoluminescence staining: Electrochemiluminescence kit	
		-Optical density: Gel Doc XR system	
		-Oxidative stress assay: NADPH oxidase activity (based on the ratio of $NADP^+$/NADPH), glutathione (Zhang et al., 2003 method), glutathione peroxidase activity (Paglia and Valentine. 1967) based on a reaction catalyzed glutathione oxidation by H_2O_2 and is coupled with the conversion of NADPH to NADPH+ and change in absorbance)	
		-Measurement of Methylglyoxal level in left ventricle: Chaplen et al., 1996 and Ma et al., 2009 Methods.	
		-Determination of N^{ε}-(carboxymethyl) lysine (CML) in left ventricle: ELISA kit	
		-NF-κB (p65) DNA-binding activity (as NF-κB activation indicator): ELISA kit	
		-In vitro antiglycation assay: Wells-Knecht et al., 1995 method.	
		-AGE formation: autofluorescence method, spectrofluorometer	
		-Collagen solubility assay: by measuring the hydroxyproline concentration after 24 h of pepsin digestion and 24 h of HCl	

		hydrolysis at 110 ◦C.	
		-protein concentration: Bradford, 1976 method	
Wistar rats (M), Age= 6	4 randomized G (6 A/G) received either 8 ml red beetroot juice (containing 79.3 mg betaxanthins + 159.6 mg betacyanins/100 ml)/kg (G: II, IV) or water (G: C, III), by gavage, daily; for 28 days. At 27^{th} day, 150 mg (nitrosodiethylamine) NDEA/kg was i.p. administered to G III and IV. After 24 h, the livers were removed from sacrificed A. Approximately 500–600 ml of juice consumed daily by an average-weight adult human.	-Preparation of microsomal and cytosolic fractions of liver: differential centrifugation	(Krajka-Kuźniak et al. 2012)
		-Protein concentrations: Lowry et al., 1951 method	
		-Phase I and phase II enzyme activity assays: ((ethoxyresorufin-O-deethylase, methoxyresorufin-O-demethylase and penthoxyresorufin-O-dependentylase: Baer-Dubowska et al., 1998 and Burke et al., 1994 method), (p-nitrophenol hydroxylase: Reinke and Moyer, 1985 protocol)), (cytosolic NAD(P)H:quinone oxidoreductase-1: Ernster, 1967 and Benson et al., 1986 methods), and (glutathione S-transferase: Habig et al., 1974 method))	
		-Cytosolic and microsomal proteins separation: Laemmli,	

		1970 method	
		-DNA damage quantification in liver homogenates: Comet assay, Hartmann et al., 2003 method	
S-D rats (F & M), W= 2003O	*In vitro* and *in vivo* physiological effects of Betanin were studied via the spontaneously active portal vein and anesthetized A, respectively. A received 4.5 µmol betanin, by gavage or intravenously. Blood, urine and feces samples was collected. Rats were anesthetized and their liver were removed. Also, 9µmol/100ml betanin was added to the perfusion fluid, and bile and blood samples were withdrawn at regular intervals. betanin breakdown was studied via incubation of pieces of gastrointestinal tract with 2.25 µmol betanin in 0.9% NaCl with or without 70% ethanol, for 24h.	-betanin: beetroot powder no E-162 (Germany) which contained about 1% betanin was suspended in phosphate buffer. This stock solution referred in this study as betanin (its betanin content being determined by its absorption at 535 nm)	(Krantz, Monier, and Wahlström. 1980)
		-Isolation of liver perfusion: Wahlstriim and Blennow, 1978 method	
		-betanin breakdown in the gastrointestinal tract: 24 h incubation with 2.25 µmol betanin in either 0.9% NaCl or 0.9% NaCl containing 70% ethanol	
		-*In vitro* physiological effects of betanin: spontaneously active portal vein for recording mechanical activity	

48 Wistar rats (M), W= 240 ± 10	6 randomized G (6 A/G) received either 8 ml red beetroot juice (containing 79.3 mg betaxanthins + 159.6 mg betacyanins/100 ml)/kg (G: II, V, VI) or water (G: C, III, IV), by gavage, daily; for 28 days. At 27th day, 150 mg (nitrosodiethylamine) NDEA/kg (G: III, V) or 2 ml carbon tetrachloride/kg (G IV and VI), was i.p. administered, and after 24 h, blood was collected from the heart of anesthetized rats and the liver homogenate was prepared.	-Liver and microsomal lipid peroxidation: by measuring thiobarbituric acid reactive substances	(Kujawska et al. 2009)
		-Measurement of reduced glutathione in liver homogenate: titration with Ellman's reagent and spectrophotometric quantification	
		-Antioxidant activity: ABTS radical cation discoloration assay	
		-Determination of protein carbonyl= kit	
		-Preparation of microsomal and cytosolic fractions of liver: differential centrifugation	
		-Protein concentrations: Lowry et al., 1951 method	
		-Superoxide dismutase activity: based on the inhibition of spontaneous epinephrine oxidation	
		-Catalase activity: by following the rate of H_2O_2 reduction	
		-Glutathione peroxidase and Reductase activity: spectrophotometer	

		-Plasma concentration of protein carbonyl: ELISA	
		-DNA damage quantification in whole blood leukocytes: Comet assay, Olive and Banath protocol, 2006	
F344, Harlan S-D rats (M), Age= 4–5	4 randomized G (18 A/G) received either 0.2 ml of 20% dimethyl sulfoxide in water as vehicle (C) or 0.3mg/kg of NMBA (N-nitroso methyl benzylamine) in-vehicle (dimethyl sulfoxide +NMBA G) or in E-162 water (NMBA+ E162-water G) or E162-water (containing 78 µg/ml commercial RBR E162 dye), s.c., 3 times weekly for 5 wks.	-Immunohistochemistry: antibodies for antigens that serve as biomarkers for proliferation (Ki-67), angiogenesis (microvessel density by CD34), inflammation (CD45), and apoptosis (terminal deoxynucleotidyl transferase dUTP nick end-labeling [TUNEL]).	(Lechner et al. 2010)
		-TUNEL staining: ApopTag Plus kit	
Post-weaning S-D rats (M), Age= 4, W= 80–100	3 randomized G received either a control diet or a supplemented diet with 10 ppm aqueous high pigment beetroot fraction IV + designated diet "A" or a supplemented diet with 150 ppm aqueous high pigment beetroot fraction I + designated diet "B", for an additional 60 days. Finally, A were sacrificed and liver, intestine, colon, kidney, and lung was excised and analyzed.	-Preparation of Crude Extracts and fractions: aquas and ethanolic extraction from lyophilized beetroot and freeze-drying of these extract (Wettasinghe et al. 2002)	(Lee et al. 2005)
		-Purification and Identification of betalains in Quinone reductase-inducing Isolates: Sephadex LH-	

		20 column and preparative Liquid Chromatography	
		-Analysis of Active Quinone reductase-inducing fractions: liquid chromatography-mass spectrometry	
		-Glutathione S-transferase activity: Habig et al., 1974 method	
		-Protein concentration: Bradford, 1976 method	
		-Enzyme assays: optical microtiter plate scanner	
		-*In Vitro* Antioxidant and Phase II Enzyme activities: ABTS radical cation discoloration assay	
		-Measurement of reducing power: based on the ability to reduce ABTS•+	
		-Inhibition of peroxyl radical-mediated β-carotene bleaching: based on the ability to inhibit decolorization of β-carotene	
		-Bioassay of phase II enzyme-inducing potency: The "Prochaska" bioassay is based on the induction of Quinone reductase-specific activity in murine hepatoma, Fahey et al., 2004 method.	
80 ICR mice (M), Age= 5–6, W= 18–22	5 randomized G (16 A/G) received no treatment (C) or 0 or 5 or 20 or 80 mg betalains from RBR (80 mg/kg was equivalent to a dose of betanin)/kg, orally, daily for 30 days. Then irradiation on experimental G (except C) was	-betalain analysis: HPLC	(Lu, Wang, and Zhang. 2009)

	performed. After irradiation, the same interventions repeated for 3 days.		
		-The activities of superoxide dismutase, glutathione peroxidase, malondialdehyde, and catalase: spectrophotometrically using kits	
		-Protein concentration: Bradford, 1976 method	
		-White blood cells count: hemocytometer	
		-Spleen and Thymus index: Uma Devi and Prasanna, 1995	
		-Bone marrow micronucleus assay: Hosseinimehr et al., 2003; Schmid, 1975	
		-Whole-body irradiation: cobalt-60-γ-radiation source	
Swiss mice (M), W= 25–35	I, II, III: 4 randomized G received either 30 or 100 or 300 mg betalain/kg or 10 ml vehicle (saline)/kg, i.p., and 2 randomized G received either 100 mg betalain (containing 5% betanin)/kg, s.c. or the same orally, 30 min before 100 µg carrageenan injection (CRG)/paw. After 3 h, Paw edema was evaluated. Also, 4 randomized G received either 100 mg betalain/kg or 10 ml vehicle/kg, i.p., 30 min after CRG, or 48.5 h after CFA (complete Freund's adjuvant injection) and paw edema was measured at 0 and 3 h after	-Production of betalain-rich dye from *Beta vulgaris*: alcohol precipitation method (INPI patent PI9802148-6), betanin concentration = 5 ± 0.3%	(Martinez et al. 2015)

	CRG or 48–72 h after complete Freund's adjuvant injection (saline= negative C of inflammation, positive C= 10 mg indomethacin/kg orally, 30 min before CRG). IV: 2 randomized G received either 100mg betalain or vehicle/kg, i.p., 30 min before 500 µg CRG/cavity. And the effect of betalain on leukocyte migration and capillary permeability were assessed 6 h after 500 µg/200 µl CRG i.p. stimulus (positive C= 2 mg dexamethasone/kg, s.c., 1 h before CRG).		
		-Paw Edema test: induction of Edema by carrageenan (100 µg/paw) or complete Freund's adjuvant (complete Freund's adjuvant injection, 10 µl/paw) injection into the plantar surface of the mice hind paw. And the paw thickness was measured with a caliper (edema/mm).	
		-Myeloperoxidase activity: kinetic-colorimetric assay via spectrophotometer (hind paw tissue of the mice were collected 5 h after injection).	
		-Leukocyte migration and capillary permeability: intravenously injection of Evan 's Blue Dye Solution	
		-Total leukocytes counts: newbauer chamber and staining with May-Grüwald Giemsa.	

		-Superoxide anion production in peritoneal fluid: nitroblue tetrazolium assay via spectrophotometer, Campanini et al. 2014 method	
		-Cytokine measurement: ELISA	
48 Wistar albino rats (M), W= 160-180	5 randomized G (5 A/G) received either normal diet + distilled water (positive C) or 100 mM carbon tetrachloride (CCl_4) without (negative C) or with either 100 or 200 or 300 µg methanolic extract of RBR/ml per os, i.p., for 14 days. And on the 15^{th} day, blood and liver samples were collected from anesthetized A.	-Antioxidant enzymes assay: DPPH scavenging assay	(Rose, Sudha, and Sudhakar. 2014)
		-Phenolics and flavonoids content: Folin-Ciocalteau assay method. Total phenolics content (mg gallic acid/g of dry mass of extract) and the total flavonoids content (mg quercetin/g of dry mass of extract).	
		-Determination of LD_{50} of CCl_4: The rats were orally fed with different doses of CCl_4 and the LD_{50} value was calculated as per the method of OECD – 423 guidelines and was 2400ml/kg $_{b.w.}$ for a period of 14 days.	
		-Hepatotoxicity induction: intraperitoneal treatment of rats with 1.9ml/kg of CCl_4 (daily)	
S-D rats (F), Age= 6, W= 240 ± 10	First of study was like (Krajka-Kuźniak et al. 2012) study, but, at 27^{th} and 28^{th} day, 10 mg 7,	-Cytosolic and microsomal fractions of liver: differential centrifugation	(Szaefer et al. 2014)

	12-dimethylbenz[a]anthracene (DMBA)/kg was i.p. administered, and after 24 h, blood was collected by heart puncture from anesthetized A.		
		-Protein concentrations: Lowry et al., 1951 method	
		-Phase I and phase II enzyme activity assays: ((ethoxyresorufin-O-deethylase, methoxyresorufin-O-demethylase and penthoxyresorufin-O-dependentylase: Baer-Dubowska et al., 1998 and Burke et al., 1994 method), (p-nitrophenol hydroxylase: Reinke and Moyer, 1985 protocol)), (cytosolic NAD(P)H:quinone oxidoreductase-1: Ernster, 1967 and Benson et al., 1986 methods), and (glutathione s-transferase: Habig et al., 1974 method))	
		-Cytosolic and microsomal proteins separation: Laemmli, 1970 method	
		-DNA damage quantification in liver homogenates: Comet assay, Hartmann et al., 2003 method	
SPF grade S-D rats (M), W=220 ± 20	4 randomized G (10 A/G) received either 25 (G III) or 100 (G IV) mg betanin/kg or the same volume of distilled water (C: I, II) by intragastric gavage, daily for 5 days. At the 3rd day following administration of betanin, rats received 20 mg/kg of paraquat (II, III, IV) or normal saline	-Serum creatinine, blood urea nitrogen, urine neutrophil gelatinase-associated lipocalin and microproteinuria: kits	(Tan et al. 2015)

	(vehicle; I), i.p. A were euthanized after 24 h. Serum, urine and Kidney samples were collected.		
		-Serum nitrate/nitrite: Griess reaction	
		-kidney injury: histopathology assay	
		-Oxidative stress assay: (superoxide dismutase activity: Beauchamp and Fridovich, 1971), (CAT activity: Claiborne, 1985), and (the lipid peroxidation: Saleem et al., 2000).	
		-Protein levels of inducible NO synthase, Cyclooxygenase-2: Western Blot analysis	
		-NF-κB (p65) DNA-binding activity (as NF-κB activation indicator): ELISA kit. nuclear protein was extracted from kidney tissue.	
		-Isolation of kidney lysosomal fraction: Wattiaux et al., 1977 method	
		-β-d-glucuronidase activity: Kawai and Anno, 1971 method	
		-β-d-Galactosidase activity: Conchie et al., 1967 method	
32 Wistar rats (M), W= 200±20	2 randomized G received either rat chow containing 2 g of lyophilized RBR/kg (n=8) or the standard rat chow (n=24) daily for 10 days. Then the rats were assigned to one of four G (8 A/G); C (appropriate samples were taken under deep	-liver concentration of the diene-conjugates: method of the Association of Official Analytical Chemists, Fukai et al., 2005 method	(Váli et al. 2007)

	narcosis without operation), sham-operated G (laparotomy was performed for 60 min without damage done to the liver), ischemia-reperfusion G (after the 45-min ischemia period, liver restored to the original hemodynamic conditions) and reperfusion G with BR pretreatment (like ischemia-reperfusion G). After 15 min of reperfusion, the livers were removed and blood samples were collected.		
		-Plasma and liver concentrations of Free SH group: Sedlak's method based on Ellmann's reaction	
		-Plasma and liver ability of H-donating: Blois's method modified by Blázovics et al in the presence of DPPH-dye (spectrophotometrically)	
		-Plasma and liver reducing power: Oyaizu, 1986 method	
		-Chemiluminescent assay: Blázovics et al. method	
		-Antioxidant status or enzymes: Randox kits, spectrophotometer	
		-Protein concentrations: Lowry et al., 1951 method	
		-Plasma concentration of element: optical emission spectrometry	
		-Total lipids extraction: Folch et al. method	
		-Preparation of the methyl esters: boron trifluoride method	

		-Fatty acid methyl ester profiles: Gas-liquid chromatography	
		-Liver formaldehyde content: over-pressured layer chromatographic separation	
Albino Wistar rats (F & M), Age= 7-8, W= 200–250	8 randomized G (6 A/G) received either 1 ml distilled water/kg (C: I, II) or 1 (G: III, VI) or 2 (G: IV, VII) or 3 ml Detroit RBR pomace extract/kg (G: V, VIII), i.p., for 7 days. 24 h before the killing, some G (II, VI, VII, VIII) received i.p. 2 ml carbon tetrachloride (CCl_4)/kg. At 8^{th} day, A were killed, and liver weight was determined.	Preparation of RBR pomace extract from *Beta vulgaris* cultivars (Detroit, Cardeal-F1, Egyptian, Bicor, and Kestrel): ultrasonic bath, extraction with aqueous ethanol solution and acetic acid, solid-phase extraction with a vacuum manifold processor with CHROMABOND C18 column	(Vulić. 2014)
		-*In Vitro* DPPH free radical scavenging assay: spectrophotometrically, Yen and Chen, 1995 method	
		-*In Vitro* Reducing power: Oyaizu, 1986 method	
		-liver Enzyme activity: xanthine oxidase: Bergmayer; 1970 method, catalase: Beers and Sizer; 1950 method, peroxidase: Simon, Fatrai, Jonas, and Matkovics; 1974, glutathione peroxidase: Beuthler; 1984 method and glutathione reductase: Goldberg and Spooner; 1983 method	
		-Reduced glutathione of the liver: Rahman, Kode, and Biswas, 2007 method	
		-lipid peroxidation of the liver: Buege and Aust; 1978 protocol	
		-Content of phenolic compounds	

		and betalain: HPLC	
64 Wistar rats (M), Age= 4, W= 103.8 ± 3.92	8 randomized G (8 A/G) received either a standard basal diet containing 8% soybean oil and 0.3% cholesterol or a dyslipidemic basal diet containing 8% lard, 0.5% cholic acid and 1% cholesterol, both accompanied with 0 or 0.3 or 1 or 3% of RBR crisps, for 4 wks. Access to diets/water was ad libitum. Blood was collected from the caval tail vein of anesthetized A, and their gastrointestinal tract was excised and weighed.	-Quantification of betalains: spectrophotometer and HPLC	(Wroblewska, Juskiewicz, and Wiczkowski. 2011)
		-The pH of the stomach, small intestine, caecum and colon contents: microelectrode and a pH/ION meter	
		-Glycolytic activity in the caecal digesta: by the release rate of p-or o-nitrophenol from their nitrophenyl-glucosides, Djouzi, Andrieux, 1997 and uśkiewicz, et al., 2002 methods	
		-The concentration of short-chain fatty acids: Gas chromatography	
		-Concentration of glucose, total cholesterol, high-density lipoprotein-cholesterol, triglycerides and activity of alanine aminotransferase and aspartate aminotransferase in the serum: kits (high-density lipoprotein-cholesterol determined after selective precipitation of low	

		and very low-density serum lipoproteins with polypropylene glycol and further removal by centrifugation)	
		-The atherogenic index: total cholesterol-high density lipoprotein/ high-density lipoprotein described by Aziz, 2009	
		-Superoxide dismutase and glutathione peroxidase activities and total antioxidant status (TAS): kits (TAS assay is based on the incubation of ABTS with peroxidase (metmyoglobin), and a radical cation production which was detect spectrophotometrically	
		-lipid extraction: Folch method	
		-Kidneys and liver total hepatic cholesterol and triglycerides: kits	
Adult S-D rats (M), W= 260-300	4 randomized G received either 0.1 ml normal saline/100 g (C) or 100 mg dissolved isoproterenol into normal saline/kg, s.c., for 3 consecutive days at an interval of 24 h, to establish AMI rat model (G II-IV). AMI rats received either 0.1 ml normal saline/100 g (G II), or 25 (G III) or 100 (G IV) mg betanin/kg, s.c., daily for 3 days. Rats were sacrificed after blood collection. Hearth samples were prepared and then infarct size of heart samples were measured.	-The activity of creatine kinase, the MB isoenzyme of creatine kinase, cardiac troponin T and lactate dehydrogenase in blood samples: ELISA kits	(Yang et al. 2016)

		-Measurement of infarct size in hearth samples: tri-phenyltetrazolium chloride staining	
		-Levels of inducible nitric oxide synthase and NF-κB proteins in heart samples: Western blotting	
		-Activities of malondialdehyde, catalase, superoxide dismutase, and glutathione in blood samples: kit	
		-Measurement of reactive oxygen species production in heart samples: kit	
		-Measurement of myeloperoxidase activity in heart samples: kits	
		-Measurement of low-density lipoprotein in homogenized hearth samples: measurement of the conjugated diene lipid hydroperoxides formation at 234 nm	

A=animals, C=control group/groups, F= female, G=group(s), ELISA: Enzyme-linked immunosorbent assay, HPLC: high-performance liquid chromatography, i.p.= intraperitoneally, i.v.=intravenously, /kg= /kg $_{b.w.}$, M=male, *OFI= Opuntia ficus indica* fruit, RBR=red beetroot, s.c.=subcutaneously, S-D= Sprague-Dawley, W= Weight (Age: weeks, Weight: g)

Table 3: *In vitro* and Cell studies about Betalains/Betalains-rich food

Cells/*In vitro*	Treatment	Methods	Ref.
Plasma LDL	LDL lipid treated with (myeloperoxidas+ glucose oxidase + glucose+ nitrite in the presence of either 0 or 0.5 or 2 or 5 or 10 µM	-betanin preparation: Butera et al, 2002, and Stintzing, 2002 methods	(Allegra, Tesoriere, and Livrea. 2007)

	betanin from red PPF, for 100min), and (myeloperoxidas+ nitrite, in the presence of either 0 or 5 µM betanin, and betanin consumption was measured before and after 2, 5 and 10 min), and (myeloperoxidas+ glucose oxidase + glucose, in the presence of either 0 or 2 or 5 or 10 µM betanin, for 100 min), and (myeloperoxidas+ nitrite, in the presence of either 0 or 5 µM betanin, for 100 min, and betanin consumption was measured during oxidation and the catalytic cycle of myeloperoxidas, with or without nitrite), and (myeloperoxidas+ nitrite, in the presence of either 0 or 5 µM betanin or its oxidation products, for 100 min) in separate studies.		
		-LDL preparation: isolated from EDTA plasma by ultracentrifugation, be free of other lipoproteins by electrophoresis on agarose gel, EDTA and salts removing by gel filtration on Sephadex G-25, and	

		proteins determination by Bio-Rad colorimetric method	
		-LDL oxidation: incubation with potassium phosphate buffer, supplemented with diethylenetriaminepentaceticacid and spectrophotometrically monitoring of the conjugated diene lipid hydroperoxides formation. (Production of nitrogen dioxide by the activity of myeloperoxidase in the presence of nitrite; the reaction mixture contained 50µg LDL protein, 50 µM nitrite, 53 nM myeloperoxidase, 310 ng/ml glucose oxidase and 0.56 mM D-glucose. In some experiments, nitrite and/or LDL were omitted. betanin was added to the reaction mixture dissolved in PBS.)	
		-betanin analysis: HPLC	
		-LDL-vitamin E consumption: HPLC	
Normal Murine macrophage (RAW 264.7 cells)	Cells were cultured in DMEM with GlutaMAXTM and treated either without or with 0 or 50 or 75 or 100 µM indicaxanthin in phosphate buffer pH 7.4, and after 1 h, incubated with 1 µg Escherichia coli 0127 lipopolysaccharide (LPS)/ml for 16 h. The other experiments were co-incubation of 100 µM indicaxanthin, 1 µg LPS/ml, and either 1 µM diphenylene iodonium or 100 µM α-tocopherol in a final 0.1% ethanol	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing et al., 2002 method	(Allegra et al. 2014a)

	concentration. C or LPS-treated cells that did not receive other treatments contained the relevant vehicle.		
		-Cell viability: MTT assay	
		-Measurement of prostaglandins (PGE_2, PGD_2, and 15D-PGJ_2): Enzyme Immunoassay Kit	
		-Protein concentration: Bradford, 1976 method	
		-Protein levels of cyclooxygenase-2, microsomal PGE_2 synthase-1, hematopoietic PGD_2 synthase and β-actin proteins: Western Blotting	
		-Cyclooxygenase-2, microsomal PGE_2 synthase-1, hematopoietic PGD_2 synthase and glucose 6-Phosphate dehydrogenase: quantitative real-time reverse-transcription PCR	
		-NF-κB luciferase assay: luciferase reporter plasmid, pTAL-NF-κB (Transfections were carried out using Amaxa Nucleofector Technology)	
		-Measurement of reactive oxygen/nitrogen species: Fluorimeter	
		-Conjugated diene hydroperoxides evaluation: spectrophotometrically	
		-HNE-protein adducts determination: ELISA Kit	
Mammary (MCF-7), prostate (PC3), colon (Caco2) and hepatic (HepG2) cancer, also normal fibroblast (NIH3T3) as C	Cells were cultured in the DMEM-F12 and treated with nine PPFJs (diluted 1:100 with DMEM-F12), separately, and their viability was measured.	-°Brix value= refractometer	(Chavez-Santoscoy, Gutierrez-Uribe, and Serna-Saldívar. 2009)
		-pH values: pH-meter	
		-Juice acidity: expressed as citric acid equivalents, AOAC procedure 942.15 based on titrating the juice with NaOH to a pH of 8.2	
		-Juice color: Color Meter	

		-Total phenolics: mg gallic acid/ L juice, Folin-Ciocalteau assay, Vinson, 2001 method	
		-Total flavonoids: mg quercetin/ L juice, colorimetric assay, Zhishen et al, 1999 method	
		-Total betalains: spectrophotometrically, Stintzing et al, 2005, Trezzini and Zrÿd, 1991 and Wyler and Meuer, 1979 methods	
		-The antioxidant capacity (ORAC): Huang et al, 2002 method, the peroxide radicals were produced by 2,2′-Azobis(2-amidinopropane) dihydrochloride, using fluorescein as substrate and Trolox as standard.	
		-Cell Viability: by CellTiter 96®AQueousOne Solution Cell Proliferation Assay	
Hepatic (Huh7), PON1-Huh7, and colorectal (HT-29) cancer	Huh7 were stably transfected with a 1009 bp fragment of the human Paraoxonase1 (PON1) promoter. Huh7, HT-29, and PON1-Huh7 cells were cultured in high glucose (4.5 g/l) Dulbecco's modified Eagle. Betanin effects evaluated on cytotoxicity (cells incubated with either 0 or 1 or 5 or 10 or 15 or 25 or 35 µM betanin for 24 h (PON-Huh7 for 48 h)), H_2O_2 induced DNA damage (HT-29 cells were incubated with either 0 or 15 µM betanin for 14 h. Following that cells	-Scavenging of DPPH-, galvinoxyl-, superoxide- and hydroxyl-radical: Electron spin resonance spectroscopy and spin trapping techniques. For evaluation of the scavenging effect of betanin by Electron spin resonance, the reaction mixture contained either 0 or 1 or 2.5 or 5 or 7.5 or 10 µM betanin and either DPPH or galvinoxyl or hypoxanthine + 5,5-Dimethyl-l-Pyrroline-N-Oxide + xanthine oxidase or FeSO4 + H_2O_2 + 5,5-Dimethyl-l-Pyrroline-N-Oxide.	(Esatbeyoglu et al. 2014)

	were stressed with 25 µM H_2O_2 for 15 min), Paraoxonase1 transactivation (PON1-Huh7 cells were incubated with either 0 or 1 or 5 or 15 µM betanin for 48 h, and 25 µM Resveratrol was positive C), Nrf2 transactivation (Huh7 cells were transiently transfected with pARE GIGPx_Luc and supplemented with either 0 or 1 or 5 or 15 µM betanin for 24 h), protein levels of Heme oxygenase-1 (Huh7 cells incubated with either 0 or 1 or 5 or 15 or 25 µM betanin for 24 h), and glutathione concentration (Huh7 cells were incubated with either 0 or 1 or 5 or 15 µM betanin for 24 h). In the last three evaluation, 5 µM sulforaphane was positive C.		
		-Cell viability: colorimetric Lactate dehydrogenase assay	
		-DNA damage quantification: Comet assay	
		-Transactivation of Nrf2 and Paraoxonase1: Transient transfection and luciferase reporter gene assay	

		-Heme oxygenase-1 protein levels: Western blot analysis, ELISA kit	
		-Glutathione assay: Vandeputte et al, 1994 method, colorimetric	
Endothelial (HUVECs) cell	Cells were cultured in supplemented MV2 and their viability was measured.in the presence of 1, 5, 10, 25 and 50 µM of betanin and indicaxanthin. Also cultured cells after 48 h starved in serum-free medium for 8 h and then incubated with 5 µM of either betanin or indicaxanthin for 5 h. Then the cells were incubated with 1 ng/mL TNF-α and/or 5 µM betalains for 16 h. Incubation in the presence of betalains and relevant controls were done in ECBM MV2 medium without ascorbic acid and FCS.	-Human umbilical vein endothelial cells were isolated from healthy, nonsmoking women.	(Gentile et al. 2004)
		-Preparation of betalains from *OFI*: methanolic extraction, Purification with gel filtration on a Sephadex G-25 column, HPLC and Quantitation with spectrophotometer (Butera et al. 2002),	
		-Cell viability: MTT assay	
		-Flow cytometry analysis of cells: fluorescence-activated cell sorting	
Prostate (PC-3), breast (MCF-7)	Cells were cultured in RPMI 1640 and treated	-Cell viability and growth: MTT assay	(Kapadia et al. 2011)

	with either 0 or 0.29 or 2.9 or 29 or 290 µg/ml of RBR extract (E162 dye) or doxorubicin for 72 h, and their viability was measured. Also, PC-3 cells were incubated with 29 µg RBR extract/ml, and cell growths after 1 to 7 days were assessed. The non-treatment Gs did not contain the RBR extract.		
Hepatic normal (THLE-2) and cancer (HepG2)	THLE-2 and HepG2 were cultured in the supplemented BEGM, and Dulbecco's modified Eagle, respectively, and then incubated with either 2 or 10 or 20 µM of betanin in 0·1% dimethyl sulfoxide or 0·1% dimethyl sulfoxide alone (C) for 72 h.	-Cell viability: MTT assay	(Krajka-Kuźniak et al. 2013)
		-Protein concentration: Lowry et al., 1951 method	
		-Nuclear and cytosolic extracts preparation: nuclear/cytosol fractionation kit	
		-Extraction of DNA and RNA: Kit	
		-Genes expression levels: Quantitative Real-time PCR	
		-Nrf-2 binding to ARE sequences assay: ELISA Kit	
		-levels of glutathione s-transferase (GSTA, GSTM, GSTP, GSTT), NAD(P)H:quinone oxidoreductase 1,	

		heme oxygenase-1, p53, Keap1, Nrf2, β-actin, ERK, JNK, AKT Proteins: Western blotting	
		-NAD(P)H:quinone oxidoreductase 1 activity: Ernster, 1967 and Benson, 1986 methods	
		-Glutathione s-transferase activity: Habig et al., 1974 method	
		-DNA methylation analysis: methylation-specific PCR	
Caco-2 cells Human hepatic (HepG2, Ha22T, HUH 7), breast (MCF7), cervix	Cells were cultured and then treated with 0 or 10 µM (for cytotoxicity measurement: 25-100 µM) indicaxanthin for 72 h.	-Cell viability: MTT assay	(Naselli et al. 2012)
		-Evaluation of DNA methylation: Methylation-Sensitive Arbitrarily-Primed PCR	
		-Apoptosis detection and quantification: flow cytometric analysis	
Caco-2 cells	Cells were cultured in Dulbecco's modified Eagle. For evaluation of cytotoxic effects; cultured cells were treated with indicaxanthin (0, 125 and 150 µM) and with *OFI* extract (0, 500 and 1000 mg of fresh pulp/mL) for 48 h. For evaluation of *OFI* extract and indicaxanthin effects; Cultured cells treated without (control) or with 115 µM indicaxanthin for	-Preparation of yellow *OFI* extract and indicaxanthin: Metanolic extraction, Quantification with HPLC-electrospray ionization mass spectrometry, Stinzing, 2002	(Naselli et al. 2014)

	different time intervals (6, 12, 24 and 48 h). For evaluation of indicaxanthin effects on methylation and activation of $p^{16INK4a}$; Cultured cells were treated with indicaxanthin (0, 10 and 50 µM) and with 10 µM 5-azacytidine (a de-methylating agent as a positive control) for 48h. For evaluation of indicaxanthin effects on CDK4 and RB levels and cell cycle distribution; Cultured cells treated without (control) or with 50µM Indicaxanthin, for 48 h.		
		-Cell viability: MTT assay	
		-Measurement of apoptosis: fluorescence-activated cell sorting	
		-Analysis of cell cycle: fluorescence-activated cell sorting	
		-Reactive oxygen species production: fluorescence changes arising from intracellular oxidation of dichloro-dihydro-fluorescein diacetate	
		-Measurement of thiols: spectrophotometrically	
		-Analysis of the CpG island of $p^{16INK4a}$ gene promoter: Methylation-sensitive restriction endonucleases multiplex-PCR (IL-4 internal region was the internal control)	
		-mRNA levels of $p^{16INK4a}$: Real-time PCR (β-actin was the internal control)	

		-Total RNA isolation: TRIzol Reagent	
		-Nucleic acid concentrations: NanoDrop Spectrophotometer	
		-Protein levels of $p^{16INK4a}$, CDK4 and Retinoblastoma protein (b-tubulin was the internal control): Western Blot analysis	
Colon cancer (HCT116, LoVo1, Caco2, HT29, DLD1)	Cells were cultured in Dulbecco's modified Eagle and then treated with 0 or 10 or 50 or 100 or 200 µM indicaxanthin, for 48 h.	-Cell viability: MTT assay	(Naselli et al. 2015)
		-Methylation analysis of long interspersed nuclear element 1: quantitative PCR	
		-Extraction of genomic DNA from all the analyzed cell lines: phenol-chloroform protocol	
		-Measurement of DNA concentration: NanoDrop spectrophotometer	
		-Measurement of DNA purity: absorbances at 260 and 280 nm	
		-Purification of DNA: QIAquick Gel Extraction Kit	
		-Measurement of long interspersed nuclear element methylation: Quantitative PCR	
		-Gene-Specific Methylation Analysis: quantified by COBRA (combined bisulfite restriction analysis) using the PCR	
		-The percentage of methylation: densitometry analysis	
		-Quantification of DNA methyltransferases and Demethylase Gene Expression: quantitative real-time PCR	
		-DNA methyltransferases Activity Assay: Spectrophotometrically by a cell-independent assay using a Kit.	
		-Molecular Modeling of indicaxanthin and DNA methyltransferases Interactions: using Data Bank and software	

Human breast (MCF-7, MDA-MB-231), colorectal (HT-29), and Mouse melanoma (B16F10) cancer and normal human fibroblasts (MRC-5) and endothelial (HUVEC) cells	Cells were cultured (2D and 3D) as monolayers in RPMI 1640 (B16F10), MEM (MCF-7, MDA-MB-231, MRC-5), DMEM (HT-29), or M199 (HUVEC) and treated with either 0 (C) or 10 or 20 or 30 or 40 μM betanin/isobetanin (isolated from RBR) for 24–48 h.	-betalain quantification and analysis: spectrophotometer ($A^{1\%}$=1120 for betanin and isobetanin (at 538nm) and 750 for betaxanthins (at 477nm) and HPLC/Electrospray Ionization-Mass Spectrometry	(Nowacki et al. 2015)
		-Total carbohydrate quantification: DuBois et al., 1956 method	
		-Protein quantification: Lowry, 1951 method and Bradford, 1976 method	
		-Cultures: Nunclon polystyrene plates were used for 2D cultures and polyhydroxy ethyl methacrylate coated polystyrene plates for 3D cultures (Velzenberger et al.,2008).	
		-Histograms analysis: Wincycle software	
		-Apoptosis detection and quantification: flow cytometric analysis	
		-Detection of DNA fragmentation: Apostain binding assay (AbCys)	
		-Apoptotic signaling pathway analysis: Human apoptosis arrays	
		-Change in the mitochondrial membrane potential: treated MCF-7 cells were resuspended in a diluted MitoCapture dye. And then detected by epifluorescence microscopy.	
		Autophagy assay: Kit	
Simulated Gastrointestinal Conditions	25 mg betalains was dissolved in 100 ml distilled water (C) and in stages I, II and III,	-betalains Powder Preparation: Ethanolic extraction and freeze drying	(Pavlov et al. 2005)

	solution pH corrected till 2.5, 5.5 and 6.5, then 0.125 g of pepsin, 2% bile salts and 0.2% pancreatin were added and the mixture was incubated at 37 ◦C for 1 h, 15 min, and 1h, respectively. Before and after each stage, samples were taken and analyzed.		
		-Acid Tolerance Experiments: in the presence of 100 ml acidified water with 4 M HCl (pH 2, 2.5, and 3) and 25 mg betalain powder	
		-Bile Salts Tolerance Experiments: in the presence of 100 ml 1%, 2%, 3%, and 4% bile salts and 25 mg betalain powder	
		-*In Vitro* Gastrointestinal Tract Experiments: described in treatment part.	
		-betalains Content: Spectrophotometrically	
		-Radical Scavenging Activity: Spectrophotometrically with of Yen and Chen, 1995 method and with Pavlov et al., 2002 method	
chronic myeloid leukemia (K562) cancer	Cells were cultured in supplemented RPMI 1640, incubated with either 0-80 µM betanin and their viability was measured. For further analysis, cells were treated with either 0 or 20 or 40 or 80 µM betanin for 24 h.	-betanin preparation: methanolic extraction, isolation with gel filtration on Sephadex-LH 20 column, Submission of fractions containing the pigment to lyophilization, purification by semi-preparative HPLC, and confirmation of structural identity of betanin by nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry	(Sreekanth et al. 2007)
		-Cell viability: MTT assay	
		-Specimens observation: Scanning/Transmission	

		electron microscopy	
		-Apoptosis detection and quantification: flow cytometric analysis using propidium iodide in Reddy et al., 2003 method.	
		-Detection of DNA fragmentation: SDS/Proteinase K/RNase A extraction method (Reddy et al., 2003).	
		-Analysis of mitochondrial membrane potential: Flow cytometric analysis, Seuduto, and Grotyohann, 1999 method	
		-Protein concentration: Bradford, 1976 method and Western blot analysis	
		-Intracellular localization of betanin in K562 cells: observation under confocal microscope	
Pooled plasma from H (6 F, 4 M)	Pooled plasma was incubated with either 0 (PBS alone) or 25 or 50 or 75 or 100 µM of either betanin or indicaxanthin (*OFI* pulp) in PBS, for 15min, followed by isolation and incubation of LDL with oxygen-saturated EDTA-free PBS, supplemented with 40 µM CuCl2 as a pro-oxidant.	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing et al., 2002 method	(Tesoriere et al. 2003)
		-Plasma separation: centrifugation	
		-Preparation of LDL: isolated from EDTA plasma by stepwise ultracentrifugation, be free of other lipoproteins by electrophoresis on agarose gel, EDTA and salts removing by gel filtration on Sephadex G-25, and proteins determination by Bio-Rad colorimetric method	
		-LDL oxidation: incubation with oxygen-saturated EDTA-free PBS, supplemented with CuCl2 as a pro-oxidant and spectrophotometrically monitoring of the	

		conjugated diene lipid hydroperoxides formation.	
		-Lag and propagation phase determination: the intercept with the extrapolations of the parts of the curve representing the lag and propagation phases.	
		-The propagation rate calculation: amount of formed conjugated diene hydroperoxides per minute, and per mg LDL protein, during the propagation phase	
		-Conjugated Diene lipid hydroperoxides analysis: extraction by CH_3Cl_3/MeOH, drying by a nitrogen stream, re-suspending in cyclohexane and quantification by spectrophotometer	
		-Consumption of Antioxidants: LDL was incubated in the presence of copper ions. Portions were taken at 0-90 min and simultaneously extracted to reveal betalains (extracted from 1 mg LDL protein with chloroform/methanol), β-carotene (extracted from 500 mg LDL protein by mixing with methanol and hexane: diethyl ether) and a-tocopherol (extracted by absolute ethanol and petroleum ether). The organic extracts were then dried under nitrogen, and re-suspended with 1% acetic acid in water (for betalains), a mixture of acetonitrile/methanol/tetrahydrofuran (for β-carotene) and methanol (for a-tocopherol), and analyzed by HPLC.	
RBC from 8 non-smoking H (5 F, 3 M), Age= 32.65 ± 10.11, BMI= 21 ± 2.0	P received a single dose of 500 g PPF pulp containing 20 mg betanin + 25 mg indicaxanthin. Blood samples were collected before (0 h) and at 1, 3, 5, and 8 h after the fruit meal. In the other experiment, blood from each participant was collected after an	-Hematocrit preparation: RBCs were obtained by centrifugation and suspended in PBS.	(Tesoriere et al. 2005)

	overnight fasting and incubated without or with either 5 or 10 or 25 µM indicaxanthin or betanin or in PBS, at 37 °C, for 15 min. Then the RBC were isolated. Finally, isolated RBCs in PBS were exposed to cumene hydroperoxide.		
		-Analysis of betalains: HPLC	
		-Oxidative Hemolysis analysis: spectrophotometer	
		-Measurement of Vitamin C and E in RBCs: HPLC	
		-Measurement of glutathione in RBCs: titration with Ellman's reagent and spectrophotometric quantification	
Oral saliva from 1 P and simulated gastric, and small intestinal digestion	P, after an overnight fasting, chewed 20 g fresh and manufactured products of red/yellow *OFI* L. Mill. (cv. Gialla and Rossa) and RBR, combined with a physiological saline solution and subsequently expelled for submission to *in vitro* simulated oral, gastric, and small intestinal digestion. In another experiment, purified betanin, vulgaxanthin I and indicaxanthin I was submitted to all phases	-Post-oral digest preparation: after an overnight fasting, the oral cavity had been rinsed with deionized water, 20 g of food sample, combined with physiological saline solution, was chewed and subsequently expelled into a tared beaker. Saline was added, and the sample was homogenized. The final pH of the preparations ranged between 4 and 4.5	(Tesoriere et al. 2008)

	of the simulated digestion and their stability compared.		
		-Post-gastric digest preparation: samples from the oral phase was transferred to an amber bottle and acidified at pH 2 with HCl, and porcine pepsin and incubated at 37 °C, for 2 h.	
		-Post-intestinal digest preparation: The pH of the remaining sample was immediately increased to 7.5 with NaHCO3, and porcine bile extract and pancreatin, an enzyme mixture of amylase, trypsin, lipase, ribonuclease, and protease, from hog pancreas, was added to the amber bottle and was incubated for 2 h at 37 °C.	
		-Preparation of the bioaccessible fraction of post intestinal digest: ultracentrifugation	
		-Extraction and analysis of betalains: methanolic extraction following with spectrophotometer and HPLC analysis	
		-betanin and indicaxanthin extraction: Butera, et al, 2002 and Stintzing, 2002 methods	
		-Isolation of vulgaxanthin I: liquid chromatography on a Sephadex G-25 column.	
		-betanidin preparation: enzymatic hydrolysis of betanin	
		-betalamic acid preparation: alkaline hydrolysis of indicaxanthin	
		-isobetanin peak identification: by the ratio R_t betanin/R_t isobetanin	
		-Peroxyl Radical Scavenging Activity of post-intestinal digest: HPLC	
		-Peroxidation of methyl linoleate: incubating with AMVN under air	
Monocyte/Macrophage (THP-1) cancer	Cells were cultured and treated with 0·1% (v/v) tetrahydrofuran as a vehicle (control) or 16	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing	(Tesoriere et al. 2013a)

	µM 7-ketocholesterol + 0·1% (v/v) tetrahydrofuran to induce apoptosis. 7-ketocholesterol was delivered to the cells using tetrahydrofuran as a vehicle. For evaluation of dose-dependent effects, cells (except control cells) were treated with 0 or 0.1 or 0.5 or 1 or 2.5 µM indicaxanthin, for 24 h. For evaluation of time-dependent effects, cells (except control cells) were treated with 2.5 µM indicaxanthin for 24 or 48 or 72 h.	et al., 2002 method	
		-Cell viability: MTT assay	
		-Cell counting: quadruplicate hemocytometer	
		-Extraction of 7-ketocholesterol: with a methanol–hexane mixture from cells treated with 16µM-oxysterol for 12 h	
		-Analysis of 7-ketocholesterol: HPLC	
		-Quantification of 7-ketocholesterol: Spectrophotometrically	
		-Cell cycle analysis: flow cytometric analysis	
		-Detection of DNA changes: Acridine orange and ethidium bromide morphological fluorescence dye staining	
		-Measurement of phosphatidylserine exposure: flow cytometric analysis	
		-Measurement of mitochondrial transmembrane potential: flow cytometric analysis	

		-Reactive oxygen species detection: flow cytometric analysis for oxidation results of 2',7'-dichlorofluorescein diacetate	
		-Measurement of cytosolic calcium: using fluo-3/AM as a fluorescent Ca^{2+} probe, which were analyzed by fluorescence-activated cell sorting	
		-Protein levels of NADPH oxidase-4, Phospho Iκ-Bα, Iκ-Bα, poly(ADP-ribose) polymerase, phospho-Bad, NF-κB, NF-κB p65 and β-actin (control): Western Blotting	
Colon cancer (Caco-2)	Cells were cultured in Dulbecco's modified Eagle under an inwardly directed pH gradient (6/7.4, AP/BL), mimicking luminal and serosal sides of the human intestinal epithelium. Trans-epithelial transport of dietary-consistent amounts of betanin and indicaxanthin from yellow and red Sicilian *OFI* L. Mill and Italian RBR, in basolateral to apical and apical to basolateral direction, was measured. Also, the effect of inhibitors (5 mM pravastatin, verapamil, 10 mM indomethacin) or substrates (10 mM ferulic acid, valproic	-Analysis of betalains: HPLC	(Tesoriere et al. 2013b)

	acid, glucose, 5 mM acetic acid) of membrane transporters on the absorption was studied. The contribution of the paracellular route was evaluated after treatment of cell monolayer with EDTA. *In vitro* digestion of betalainic food was performed for providing a post-intestinal fraction containing bioaccessible pigments.		
		-Computation of Molecular descriptors of the betalains (ClogP and ClogD): Qikprop 3.1 predict program	
		-non-polar surface/molecular surface/polar surface area analysis: CODESSA PRO software	
		-pKa values of indicaxanthin: Semi-empirical calculations by Marvin Sketch 5.0.6.1 prediction program and The Hammet and Taft linear free-energy relationships calculation by Epik 1.6 software	
		-Cell viability: MTT assay	
		-Transport experiments: using Transwell^{R} polycarbonate membrane	
		-The integrity of Caco-2 cell monolayers: by measuring the transepithelial electric resistance, using a Millicell-ERS voltohmeter.	
		-Concentration-dependent trans-epithelial transport of betalains: by varying the concentrations between 100 µM and 2 mM	
		-The flux of 5 mM phenolsulfonphthalein (phenol red) and 100 µM testosterone: spectrophotometer and	

		HPLC, respectively.	
		-The effect of purified betalains/food bioaccessible fraction on the barrier integrity of cell monolayers: trans-epithelial electric resistance values	
		-The apparent permeability coefficients (Papp): equation	
		-Efflux rate of betanin: equation	
Colon cancer (Caco-2)	Cells were cultured in Dulbecco's modified Eagle and then treated with 25 ng IL-1β/ml at 37◦C and either with 0 (one of C) or 1 or 5 or 10 or 25 µM indicaxanthin (methanol extract from the yellow *OFI* pulp) for 24 h. Another C was treated with neither IL-1β nor indicaxanthin.	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing et al., 2002 method	(Tesoriere et al. 2014)
		-Cell viability: Trypan Blue Exclusion method	
		-Evaluation of the treatments effect on tight junction permeability of intestinal epithelia: IL-1β was added on the basolateral side and indicaxanthin on the apical/luminal side.	
		-Assay for IL-6 and IL-8: ELISA	
		-Arachidonic acid cascade activity: through quantification of PGE_2 production using an Enzyme Immunoassay Kit	
		-Nitrite assay: by Griess reaction and a sodium nitrite serial dilution standard curve.	
		-Reactive oxygen species detection: flow cytometric analysis for oxidation results of 2',7'-dichlorofluorescein diacetate	
		-Assay for total reduced thiols: Spectrophotometrically	
		-Levels of IkB-a, phospho-IkB-a, NF-kB, poly	

		(ADP-ribose) polymerase proteins and NADPH oxidase-1 activity: Western blot analysis	
		-Analysis of indicaxanthin uptake in cell: HPLC, Spectrophotometrically	
-RBC from 5 non-smoking H, Age= 32.65± 10.11, BMI= 21±2	-P after an overnight fasting consumed 500 g PPF meal (methanol extract of yellow Sicilian *OFI* L. Mill). Blood samples were collected, at 0, 1, 3, 5, and 8 h post-supplementation. RBCs were obtained and incubated with either 0 or 1 or 2.5 or 5 µM indicaxanthin for 1 h, and then with a 20µM mixture of oxysterols (The mixture of oxysterols at final concentrations of 7µM-7-KC, 2µM-TRIOL, 4µM-a-epox, 1µM-7a-OH, 2µM-7b-OH, and 4µM-b-epox) for 48 h. Other RBCs treated only with 5 µM indicaxanthin for 1 h. The mixture of oxysterols was added to the cells using 0·1% (v/v) tetrahydrofuran. RBCs which incubated only with the solvent were used as C.	-indicaxanthin preparation: methanolic extraction from yellow *OFI*, isolation with liquid chromatography on Sephadex G-25, Submission of fractions containing the pigment to cryodesiccation, and purification by semi-preparative HPLC, stintzing et al., 2002 method	(Tesoriere et al. 2015)

-Normal endothelial (HUVEC)	-HUVECs were cultured in MV2 and washed with 300 ml Ringer solution (0·4% hematocrit). Then treated RBCs with or without 5 µM indicaxanthin and with or without oxysterols (see above), in the same solution was layered on HUVEC and incubated at 37◦C for 30 min, and at room temperature for 1h with 200 ml SDS (0·5%).	-RBCs isolation: Ficoll gradient	
		-Measurement of phosphatidylserine externalization and forward scatter: Fluorescence-activated cell sorting analysis using flow cytometer	
		-Measurement of cytosolic calcium: calcium-dependent fluorescence intensity was measured by fluorescence-activated cell sorting analysis	
		-Measurement of prostaglandin E_2: Enzyme Immunoassay Kit	
		-Reactive oxygen species detection: flow cytometric analysis for oxidation results of 2',7'-dichlorofluorescein diacetate	
		-Measurement of glutathione: titration with Ellman's reagent and spectrophotometric quantification	
		-Analysis of indicaxanthin partition in erythrocytes: HPLC	
		-Percentage of erythrocytes adhering to HUVEC: calculated from the absorbance ratio of Hb after incubation to Hb of erythrocytes layered on HUVEC, with correction for the absorbance of wells containing only HUVEC.	
PMN from 15 obese F,	PMNs were cultured in	-Isolation of PMNs: Density gradient medium	(Zielińska-Przyjemska et al. 2009)

Age= 45 ± 9, BMI> 30 and 9 H F (C), Age= 29±11, BMI = 22.2± 1.6	RPMI 1640 medium and treated with either 0.1 or 1 or 10% of RBJ or chips containing 1.596 g betacyanins + 0.793 g betaxanthins/L, for 30 min.		
		-Cell viability: MTT assay kit	
		-Chemiluminescence measurements: luminometer	
		-Intracellular reactive oxygen species detection: flow cytometric analysis for oxidation results of 2',7'-dichlorofluorescein diacetate	
		-Caspase-3 activity assay: kit	
PMN from 11 H (5 F, 6 M)	PMNs were cultured in RPMI 1640 and treated with either 0 (C, PBS alone) or 2 or 20 or 100 or 200 or 300 or 400 or 500 µM betanin and with 1 or 200 nM phorbol 12-myristate 13-acetate to induce oxidative stress, for 2 or 24 h. One of C group was stimulated with no phorbol 12-myristate 13-acetate.	-Isolation of PMNs: Density gradient medium (Gradisol G)	(Zielińska-Przyjemska et al. 2012)
		-Cell viability: MTT assay	
		-Chemiluminescence measurements: luminometer	
		-Reactive oxygen species detection: flow cytometric analysis for oxidation results of 2',7'-dichlorofluorescein diacetate	
		-Measurement of H_2O_2 concentration: a modification of ferrous oxidation-xylenol orange (FOX) assay (Jiang et al., 1990)	
		-DNA damage quantification: Comet assay	

		-Caspase-3 activity: Fluorometric method	
		-Procaspase-3 cleavage: Western blot analysis	
-PMN from 15 P with ulcerative colitis and 12 P with Crohn disease, Age= 42 ± 12	-Caco-2 cells were cultured in DMEM, incubated with either 0 (C) or 20 or 100 or 200 μM betanin for 24 h, and exposed to 0 (unstimulated) or 100 μM H_2O_2 for 30 min, to induce DNA damage and apoptosis.	-PMNs isolation: density gradient medium (Gradisol G)	(Zielińska-Przyjemska et al. 2016)
-Colon cancer (Caco-2)	-PMNs were cultured in RPMI 1640, incubated with either 0 (C, only PBS) or 100 or 200 μM betanin, and exposed to 0 (unstimulated) or 200 nM 12-O-tetradecanoyl phorbol 13-acetate, for 2 or 24 h, to induce DNA damage and apoptosis.	-Cell viability: MTT assay	
		-DNA damage quantification: Comet assay	
		-Mitochondrial transmembrane potential: Mitochondrial Staining Kit with fluorescent dye	
		-Caspase-3 activity: Fluorometric method	
		-Levels of Caspase-3 and β-actin proteins: Western blot analysis	
		-Disease progress assessment: Crohn's Disease Activity Index and the Ulcerative Colitis Symptoms Score	
PBMC (peripheral blood mononuclear cells) from H	Cells were cultured in RPMI 1640, preincubated without or with the RBR extract 0 or 0.1% or 1% or 5%,	-Measurement of tryptophan and kynurenine concentrations: HPLC	(Winkler et al. 2005)

	for 30 min, and stimulated or not with 10 µg/ml of concanavalin or phytohaemagglutinin-A for 48 h. unstimulated cells were used as C.		
		-Neopterin concentrations: ELISA	
		-Isolation of PBMC: density centrifugation	

cancer= cancer cell line, C =control, ELISA: Enzyme-linked immunosorbent assay, H=healthy participant, HPLC: high-performance liquid chromatography, *OFI= Opuntia ficus-indica* fruit, P=participant, PCR= Polymerase chain reaction, PMN= polymorphonuclear leukocytes/cells, PPF= prickly pear fruit, PPFJ= prickly pear fruit juice, RBC=red blood cells, RBR= red beetroot, Medium words was removed (Age: years, BMI: kg/m^2)